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CONTENTS

NUMBER 1, FEBRUARY 1961

	PAGE
Diauxic Growth Curves of Seeds, with Special Reference to French Beans (<i>Phaseolus vulgaris</i> L.). By D. J. Carr and K. G. M. Skene	1
A Quantitative Study of the Gibberellin Content of Seeds of <i>Phaseolus vulgaris</i> at different Stages in their Development. By K. G. M. Skene and D. J. Carr ..	13
Ionic Relations of Cells of <i>Chara australis</i> R. Br. IV. Membrane Potential Differences and Resistances. By A. B. Hope and N. A. Walker ..	26✓
<i>Peronospora tabacina</i> in Tobacco: Transpiration, Growth, and Related Energy Considerations. By I. A. M. Cruickshank and N. E. Rider	45
Germination of <i>Peronospora tabacina</i> : Effect of Temperature. By I. A. M. Cruickshank	58
A Thiamine-requiring Mutant of the Tomato. By J. Langridge and R. D. Brock ..	66
Additional Resistance in <i>Triticum vulgare</i> to <i>Erysiphe graminis tritici</i> . By A. T. Pugsley	70
Possible Differentiation in the Wild Population of <i>Oenothera organensis</i> . By R. A. Fisher	76
Studies on the Sodium-Potassium Balance in Erythrocytes of Australian Merino Sheep. I. Changes in Concentrations in the Erythrocytes of Lambs from Birth to 98 Days. By Judith H. Koch and Helen Newton Turner ..	79
Electrolyte and Haematocrit Changes in the Blood of Sheep from Foetal to Postnatal Life. By J. V. Evans and M. H. Blunt	87
Variation in the Gene Frequencies of Potassium and Haemoglobin Types in Romney Marsh and Southdown Sheep Established away from their Native Environment. By J. V. Evans and M. H. Blunt	100
On the Mechanism of Incorporation of [³⁵ S]Cystine into Wool. By A. M. Downes ..	109
Studies on the Rate of Wool Growth using [³⁵ S]Cystine. By A. M. Downes and A. G. Lyne	120
Studies on Experimental Dermal Cysts in Sheep. By G. S. Molyneux and A. G. Lyne	131
The Postnatal Development of Wool Follicles, Shedding, and Skin Thickness in Inbred Merino and Southdown-Merino Crossbred Sheep. By A. G. Lyne ..	141
Microscopic Analysis of Faeces, a Technique for Ascertaining the Diet of Herbivorous Mammals. By G. M. Storr	157
Pathology of Infestation of the Rat with <i>Nippostrongylus muris</i> (Yokogawa). VI. Absorption <i>in vivo</i> from the Distal Ileum. By L. E. A. Symons ..	165

NUMBER 2, MAY 1961

	PAGE
Studies of Dormancy in the Seeds of Subterranean Clover (<i>Trifolium subterraneum</i> L.). II. The Interaction of Time, Temperature, and Carbon Dioxide during Passage out of Dormancy. By L. A. T. Ballard ..	173
The Transmission of Cauliflower Mosaic Virus by Aphids. By M. F. Day and D. G. Venables	187
Environment and Sporulation in Phytopathogenic Fungi. II. Conidia Formation in <i>Peronospora tabacina</i> Adam as a Function of Temperature. By I. A. M. Cruickshank	198
Dissemination of Conidia of <i>Peronospora tabacina</i> Adam. By A. V. Hill ..	208
Studies concerning the Inheritance of Ascospore Length in <i>Neurospora crassa</i> . I. Studies on Large-spored Strains. By B. T. O. Lee and J. A. Pateman ..	223
Bioelectric Oscillations of Bean Roots: Further Evidence for a Feedback Oscillator. I. Extracellular Response to Oscillations in Osmotic Pressure and Auxin. By I. S. Jenkinson and B. I. H. Scott	231 ✓
Serum β -Globulin Polymorphism in Mice. By G. C. Ashton and A. W. H. Braden	248
On the Amino Acids Essential for the Tissues of the Sheep. By A. M. Downes ..	254
Studies on the Sodium-Potassium Balance in Erythrocytes of Australian Merino Sheep. II. Observations on Three Merino Strains. By Helen Newton Turner and Judith H. Koch	260
Differences in the Concentration of Potassium and the Type of Haemoglobin between Strains and Sexes of Merino Sheep. By J. V. Evans	274
A Comparative Study of the Monolayers of Various Cereal Proteins and of Wheat Gluten of Differing Characteristics. By N. W. Tschoegl ..	288

NUMBER 3, AUGUST 1961

	PAGE
The Quantity of Water in the Cell Wall and its Significance. By D. F. Gaff and D. J. Carr	299 ✓
Ionic Relations of Cells of <i>Chara australis</i> . V. The Action Potential. By A. B. Hope	312 ✓
The Cultivation of Isolated Roots of Subterranean Clover and Effects of Amino Acids on their Growth Pattern. By the Late P. L. Goldacre and H. Unt	323
Studies on Phytoalexins. III. The Isolation, Assay, and General Properties of a Phytoalexin from <i>Pisum sativum</i> L. By I. A. M. Cruickshank and Dawn R. Perrin	336
The Growth of <i>Rhizobium</i> in Synthetic Media. By F. J. Bergersen ..	349
The Direct Assay of ^{14}C in Dried Plant Materials. By T. P. O'Brien and I. F. Wardlaw	361
The Freezing of Plant Tissue. By D. C. Marshall	368
Studies in Translocation. I. The Respiration of the Phloem. By Margaret D. Duloy and F. V. Mercer	391
Accommodation of Gene-Chromosome Configuration Effects in Quantitative Inheritance and Selection Theory. By B. Griffing	402
Abnormal Inheritance of the Sex-linked Tabby Gene. By B. M. Kindred ..	415
The Action of Urea on Diapause in Eggs of <i>Acheta commodus</i> (Walk.) (Orthoptera: Gryllidae). By T. W. Hogan	419
The Fate of Intravenous Doses of Free and Plasma Protein-bound [^{35}S]Cystine in the Sheep. By A. M. Downes	427
A Morphological and Histochemical Study of the Bacterial Degradation of Wool Fibres <i>in vivo</i> . By G. S. Molyneux	440
The Absorption of Ammonia through the Rumen of the Sheep. By J. P. Hogan	448
Studies on Oxidized Wool. IV. Fractionation of Proteins Extracted from Wool on DEAE-Cellulose using Buffers containing 8M Urea. By I. J. O'Donnell and E. O. P. Thompson	461
Preparation of Pure Proteins from Hog Thyroid Glands by Column Chromatography on Diethylaminoethyl-cellulose. By S. Shulman and P. G. Stanley	475

Short Communications

Estimates of Cortical Differentiation in Normal and "Doggy" Merino Wools. By G. Jones	485
The Visual Pigment of an Isopod Crustacean. By M. H. Briggs.. ..	487
The Coupling of Cellobiase and Peroxidase by Glucose Oxidase. By M. A. Jermyn	489

NUMBER 4, NOVEMBER 1961

	PAGE
The Control of Tillering in the Barley Plant. I. The Pattern of Tillering and Its Relation to Nutrient Supply. By D. Aspinall	493
Studies in Translocation. II. Submicroscopic Anatomy of the Phloem. By Margaret Duloy, F. V. Mercer, and Nele Rathgeber	506
Effects of Several Osmotic Substrates on the Water Relationships of Tomato. By R. O. Slayter	519
The Electric Double Layer and the Donnan Equilibrium in Relation to Plant Cell Walls. By J. Dainty and A. B. Hope	541
Distribution of Growth and Enzyme Activity in the Developing Grain of Wheat. By A. H. G. C. Rijven and R. Cohen	552
The Nitrogen Requirements of some Members of the Viridans Group of Streptococci. By Jean I. Paul	567
The Influence of Testosterone Treatment on the Development of the Bursa of Fabricius in the Chick Embryo. By N. L. Warner and F. M. Burnet ..	580
Inheritance of Antibody Response. IV. Heritability of Response to the Antigens of <i>Rhizobium meliloti</i> and Two Strains of Influenza Virus. By W. R. Sobey and K. M. Adams	588
Inheritance of Antibody Response. V. Correlated Antibody Responses to Various Related and Unrelated Antigens. By K. M. Adams and W. R. Sobey	594
The Distribution of Larvae of Randomly Moving Insects. By E. J. Williams ..	598
Inheritance of DDT-resistance Involving the Y-Chromosome in the Housefly (<i>Musca domestica</i> L.). By R. W. Kerr	605
Growth of the Mouse Coat. VIII. Changes in the Coat and Body Weight under Heat Stress. By T. Nay	620
A Maternal Effect on Vibrissa Score due to the Tabby Gene. By B. M. Kindred	627
The Viability of Fowl Spermatozoa in Dilute Suspension. By R. G. Wales and I. G. White	637
Studies on Marsupial Nutrition. III. The Copper-Molybdenum-Inorganic Sulphate Interaction in the Rottneest Quokka, <i>Setonix brachyurus</i> (Quoy & Gaimard). By S. Barker	646
Mitotic Activity in Wool Follicle Bulbs. By P. G. Schinckel	659
Chemical Changes in Wool Treated with Solutions of Iodine. By W. G. Crewther and L. M. Dowling	677
Chromatographic Fractionation of the Acetic Acid Soluble Proteins of Wheat Flour on Carboxymethyl-cellulose. By D. H. Simmonds and D. J. Winzor	690
<i>Short Communication</i>	
Distribution of Glutamyl Transferase in the Ripening Pea Seed. By A. H. G. C. Rijven	700
Index to Volume 14	703

DIAUXIC GROWTH CURVES OF SEEDS, WITH SPECIAL REFERENCE TO FRENCH BEANS (*PHASEOLUS VULGARIS* L.)

By D. J. CARR* and K. G. M. SKENE†

[Manuscript received July 1, 1960]

Summary

The results of growth studies of pods and seeds of a variety of French beans, *Phaseolus vulgaris* L., are reported. Pod growth commences immediately after anthesis and is completed in 16–17 days. Seed growth begins about 9 days after anthesis and, irrespective of whether fresh weight, dry weight, or length is measured, is diauxic, two phases of high growth rate being separated by a lag phase. During the phases of high growth rate, growth is initially exponential but eventually declines. The lag phase lasts about 3 days, from about the 20th to the 23rd day.

An examination of the literature shows that diauxic growth probably occurs in many seeds, although few studies have been sufficiently detailed to reveal it. At the onset of the lag phase extensive modifications are initiated in the metabolism of the seed and fruit, leading to the changes associated with seed maturation, such as a fall in sucrose in pea seeds and of water content of bean pods, which have been widely reported in the literature. It is suggested that mechanical restriction imposed by the surrounding structures on further growth of the embryo causes the growth rate of the seed to fall during the lag phase.

I. INTRODUCTION

Reports of the presence of gibberellins in mature and immature seeds (reviewed by Brian 1959) have led to speculation on the possible role of these hormones in the growth and development of fruits and seeds (Luckwill 1959). Much is known of the development and growth of fruits (Nitsch 1953; Luckwill 1959) but few intensive studies have been made of the growth of seeds and embryos, although these are thought to be among the main sites of synthesis of the auxins and possibly also of the gibberellins which may be involved in fruit growth. Work has been initiated in this Laboratory on the pattern of changes of gibberellin activity in seeds during their development and the correlation of these changes with the growth and development of seeds. In this paper the results of some studies on the growth of French bean seeds will be presented, and in the following paper (Skene and Carr 1961) information on patterns of changes in gibberellin activity during the development of these seeds. Apart from some studies of the early stages of embryo development (e.g. Brown 1917) no detailed account of the growth of the bean seed is available, but the current impression seems to be that all seeds have a simple sigmoid pattern of growth. Nitsch and Nitsch (1955) have made some studies on the "auxins" of bean seeds. More recently, the isolation from seeds of *Phaseolus multiflorus* of gibberellin A₁ (MacMillan and Suter 1958) and of gibberellin A₅ (MacMillan, Seaton, and Suter 1959) has been

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reported, while similar or possibly identical substances have been isolated from seeds of *P. vulgaris* (West and Phinney 1959).

II. MATERIALS AND METHODS

Beans (*Phaseolus vulgaris* cv. Hawkesbury Wonder) were grown in soil in the greenhouse. Flowers were tagged on the day of anthesis and up to 60 pods were sampled at each 3-day interval over a period of 35 days from anthesis. Initially the pods grow much more rapidly than the ovules and ovule and seed growth has been followed only from day 8 onwards. As observed by Nitsch and Nitsch (1955) the ovules grew rapidly from the ninth day. Data from successive crops grown in the summer and autumn of 1959-60 were highly reproducible.

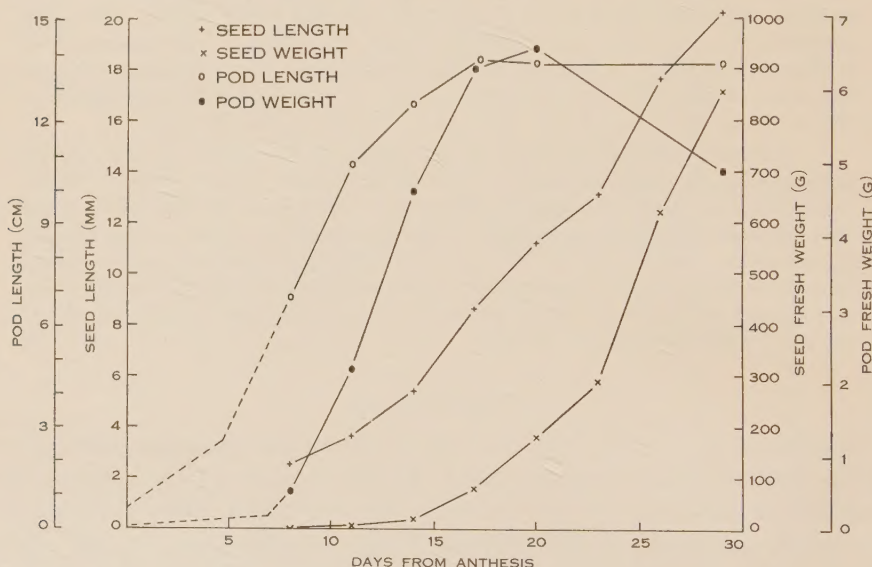


Fig. 1.—Pod and seed growth curves of Hawkesbury Wonder beans from anthesis to a stage approaching maturity of the seeds.

III. RESULTS

(a) Growth of the Pods

The pods began to grow about the second day after anthesis (as was found also by Nitsch and Nitsch 1955) and the maximum growth rate of the pods occurs in the period from then until day 11 (Fig. 1). The pods attain their maximum length between 12 and 17 days after anthesis. The curve of increase in fresh weight follows much the same course as the curve of increase in length, but the maximum length, once attained, remains constant until maturity whereas the fresh weight begins to decline before the seeds attain their maximum length.

(b) Growth of the Seeds

During the period from 8 to 20 days after anthesis the fresh weight of the seeds increases exponentially so that the relative growth rate is constant (Fig. 2). At about

20 days the relative growth rate falls and the incremental growth curve (Fig. 3) shows that the rate of growth, whether expressed as fresh weight or as dry weight, is beginning to decline. The fall in the rate of increase of weight is transient and lasts only about 3 days. This brief lag period is followed by a period of rapid growth lasting for about 3 days after which the rate of growth again declines, finally to zero. At about 30 days after anthesis the seeds begin to dry out and become mature. The pods have already begun to dry out at about 20 days after anthesis, i.e. at about the time of onset of the lag period in the growth of the seeds. Changes in the seed length (Fig. 1) show the same diauxic pattern of growth as the fresh or dry weight data.

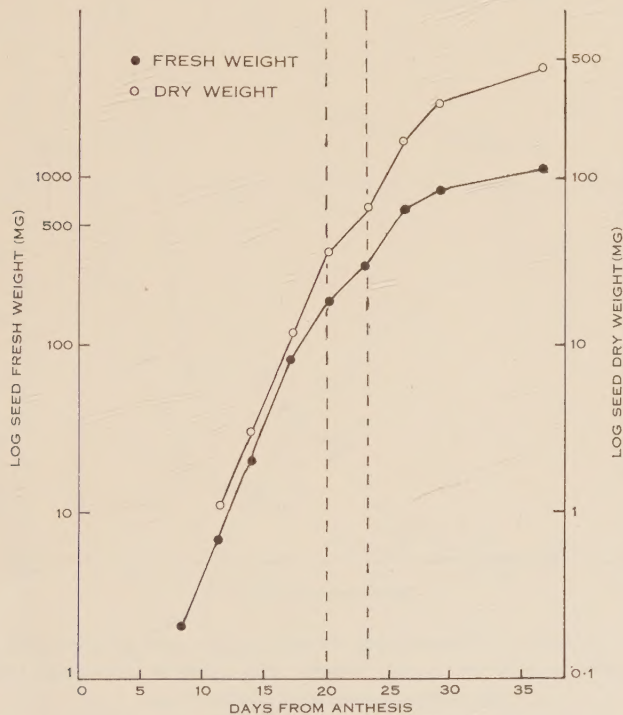


Fig. 2.—Growth of Hawkesbury Wonder bean seeds plotted as logarithms of fresh weight or dry weight against time from anthesis.

(c) Embryonic Axis

Early growth of the embryo is mainly of the cotyledons. The embryonic axis (embryo minus cotyledons) remains small until about the 23rd day after anthesis, i.e. until the end of the lag phase (Fig. 4). It then begins to grow very rapidly. The increment curve of Figure 4 shows that the most rapid growth of the embryonic axis is at about 25–26 days after anthesis which is the time of the maximum growth rate of the seed during the third phase of growth. By 35 days after anthesis the growth rate of the embryonic axis has declined considerably.

IV. DISCUSSION

The growth curve of the seeds was expected to be sigmoid, and it was rather a surprise to find that, like the growth curves of many fleshy fruits (Luckwill 1959), it was diauxic. Three phases may be distinguished in the growth of bean seeds. In the first, growth is exponential and dry weight and fresh weight increase at about the same relative rate (Fig. 2). In the second phase, lasting only about 3 days, growth, whether in length, fresh weight, or dry weight is much less rapid than in the first

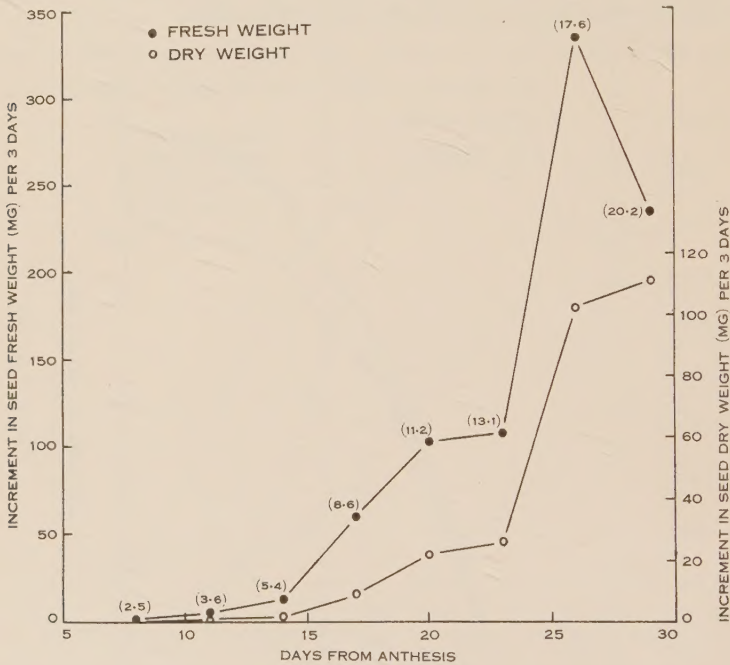


Fig. 3.—Growth of Hawkesbury Wonder bean seeds as increment (mg/3 days) of fresh weight or dry weight. The values in parenthesis refer to the corresponding seed lengths (mm).

phase: this phase will be referred to as the lag phase. In the third phase growth is initially about as rapid as in the exponential first phase, but gradually declines to zero. A search of the literature reveals that others have observed similar phases in the growth of seeds, but have ignored them in interpreting their own data.

McKee, Robertson, and Lee (1955) have published data on the growth of pea seeds which have been replotted (Fig. 5) as increment in dry weight per day. Since these authors were chiefly concerned with biochemical changes associated with the optimum harvest date for canning, their data do not extend to full maturity of the seeds, but they do show quite clearly the period of about 3 days (32–35 days from anthesis) during which the growth rate remains at a very low value, having fallen from the highest value of the previous exponential phase. In their paper these authors have drawn smooth curves through the points, thus obscuring the lag phase

which the data (for instance of their Table 1) clearly show. A lag period between days 33 and 36 after anthesis appears even more clearly in the data for Dwarf Telephone peas of Bisson and Jones (1932) when these are replotted (Fig. 6).

The data of Hyde, McLeavey, and Harris (1959) on the growth of red and white clover seeds have been replotted as Figure 7. These curves also show a 3-day period of low growth rate separating two periods of high growth rate. Up to 10 days after anthesis growth is exponential. Then there is a marked fall to a very low growth rate.

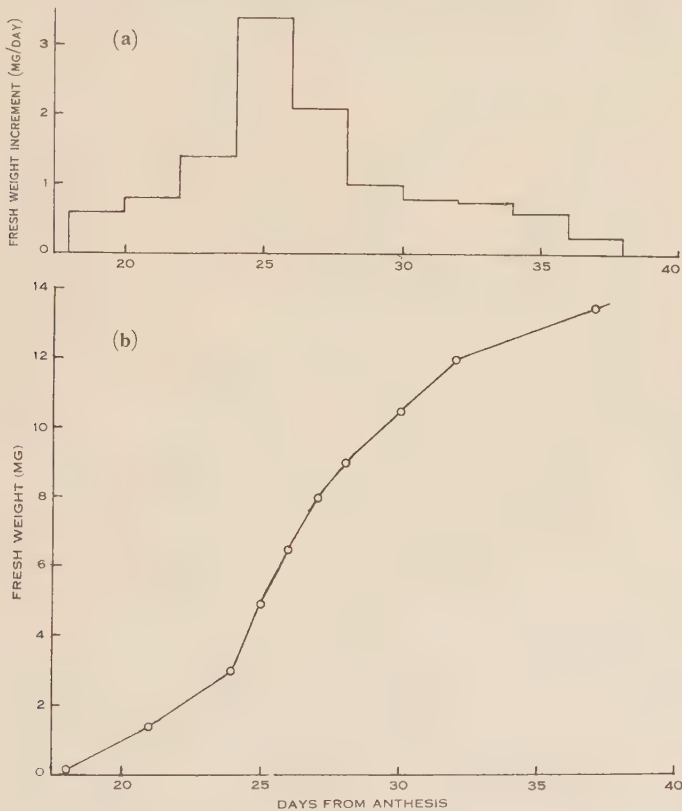


Fig. 4.—Growth of embryonic axis (embryo minus cotyledons) of Hawkesbury Wonder beans. (a) Increment (mg/day) plotted against days from anthesis; (b) fresh weight (mg) plotted against days from anthesis. Growth is maximal between 24 and 26 days from anthesis.

The final phase is itself approximately sigmoid. These data on clover seeds were collected in different years, but apparently this did not affect the general pattern of growth. In crops of Hawkesbury Wonder beans grown at different times of the year only slight and quantitative differences in the growth pattern of the seeds have been observed in our experiments. This must mean that the pattern of the growth curve is but little affected by climatic conditions, so long as these are not inimical to growth.

Other instances of diauxic growth curves of seeds are shown in the data of Garner, Allard, and Foubert (1914) on soybeans, and that they are not confined to

leguminous seeds is shown by the data of Woodroof and Woodroof (1927) on pecan nut kernels (Fig. 8). Many authors who have studied the growth of seeds have either failed to relate their data to the date of anthesis or of pollination, or have sampled at insufficiently frequent intervals (e.g. Dillman (1928) for flax seeds; Reeves and Valle (1932) for cotton seeds; Brooks (1940) for almonds). Finally there are some instances where the data are complete, but there is no clear evidence of a diauxic growth curve. The growth curve of the single-seeded caryopses of grasses, including rye grasses (*Lolium perenne* and *L. multiflorum*) (Hyde, McLeavey, and Harris 1959), rye (Nutman 1941), barley (Harlan 1920; Martini, Harlan, and Pope 1930), and wheat (Koblet

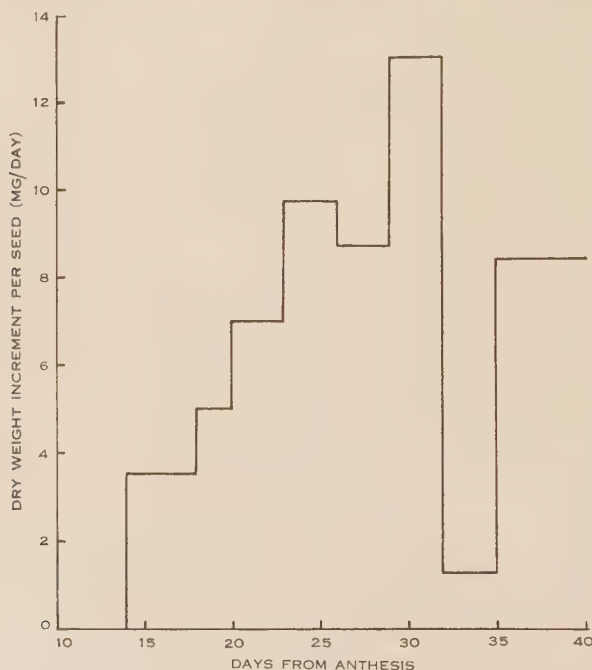


Fig. 5.—Growth of Canner's Perfection peas as increment (mg/day) of dry weight plotted against time from anthesis (from data in McKee, Robertson, and Lee 1955).

1940) is simply sigmoid. In Petkus rye the growth of the embryo sac is diauxic, with a lag period from the sixth to the ninth day after anthesis, but the embryo grows exponentially from inception up to about 22 days after anthesis, when growth of the whole caryopsis practically ceases (Nutman 1939). The curve of growth, as measured by fresh weight, of the endospermic seeds of *Datura stramonium* (Rietsema *et al.* 1955) shows no lag period, although there is a period of about 4 days (beginning 11 days after anthesis) when the rate of increase of dry weight of the ovules falls to a level much below that of the preceding and subsequent periods of growth.

The morphological explanation of the lag phase in bean (and pea) seed growth is relatively simple. In bean ovules about 4 mm long the embryo is quite small and situated at the micropylar end of the embryo sac, which is filled with a mucilaginous

substance. According to Brown (1917) the endosperm in bean ovules is a single layer of cells which soon break down and are resorbed at about 10–12 days after anthesis (i.e. in Hawkesbury Wonder beans when the ovules are about 4 mm long). Netolitzky (1926) says that the endosperm, which forms only incomplete cell walls, becomes slimy. Some doubt exists, therefore, as to the morphological nature of the contents of the embryo sac after endosperm breakdown. However, the embryo sac, with its mucilaginous contents, continues to enlarge as does also the embryo but the latter

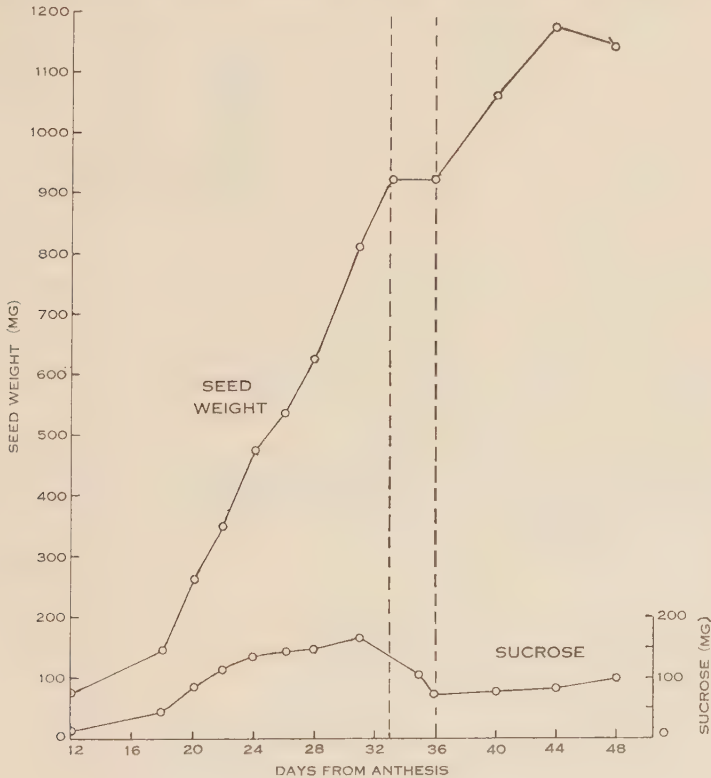


Fig. 6.—Growth of Dwarf Telephone pea seeds (mg) plotted against time from anthesis (from data of Bisson and Jones 1932). The lower curve shows the corresponding content of sucrose (mg). The lag phase in growth is enclosed by the vertical lines through days 33 and 36 from anthesis.

enlarges faster than the embryo sac. Finally, when the ovule is about 9–11 mm long, the embryo has grown until it just fills the embryo sac (Fig. 9). From this time onwards, then, both embryo and seed must grow in volume at the same rate, unless the testa is to become thinner. In bean seeds the ratio of embryo fresh weight to seed fresh weight increases up to this time then remains more or less constant thereafter. The embryo/seed fresh weight ratio for a variety of peas is shown in Figure 10. In these peas the embryo/seed ratio approaches a value of about 0.8 when the seeds are about 0.5–0.6 g fresh weight. This corresponds (in this variety) to the weight

at a time when the lag period is just commencing. The data of Figure 10 were obtained from a small-seeded variety obtained in Melbourne. In Dwarf Telephone peas (Bisson and Jones 1932) the seed weight at the onset of the lag period is 0.92 g and in Canner's Perfection (McKee, Robertson, and Lee 1955) it appears to have been about 0.47 g.

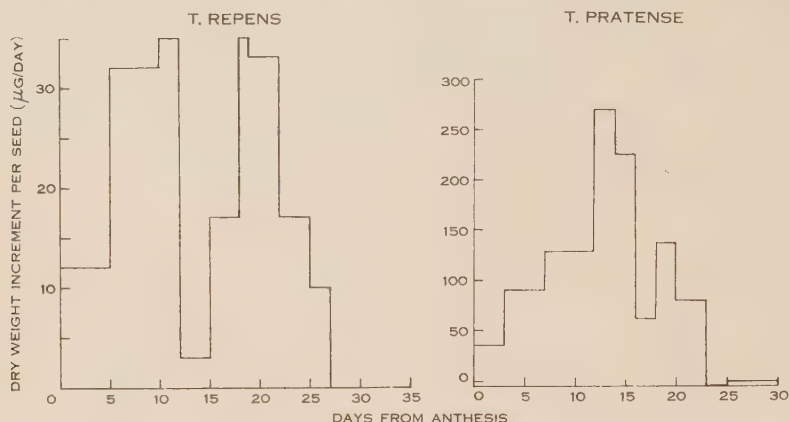


Fig. 7.—Growth increment curves ($\mu\text{g/day}$) of seeds of white clover (*Trifolium repens*) and red clover (*T. pratense*) plotted against time from anthesis (from data in Hyde, McLeavey, and Harris 1959).

It seems probable, therefore, that the seed size at which the maximum embryo/seed ratio is attained differs between varieties, but it may be somewhat independent of

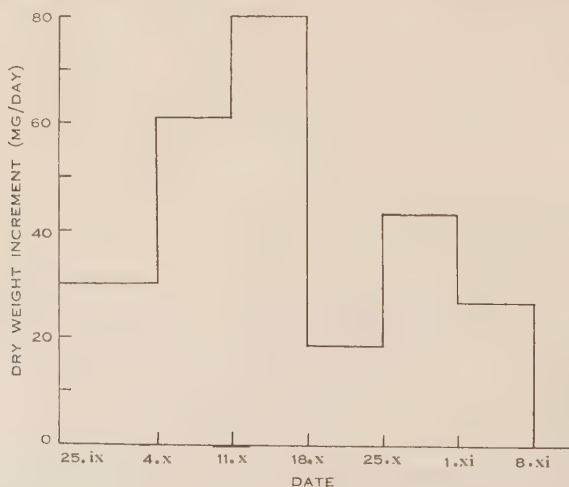


Fig. 8.—Growth increment curve (mg/day) of kernels of *Hicoria pecan* cv. Bradley (from data in Woodroof and Woodroof 1927).

small climatic fluctuations. It is probably determined by the allometry of seed and embryo growth rates. Many instances are known of gene-controlled differences in allometric growth rates (Emerson 1910; Kaiser 1935; Hammond 1941).

Figure 11 shows the sudden change in allometry of French bean seed and embryo growth at the onset of the lag phase. Before the lag phase the allometric coefficient (log seed weight/log embryo weight) is less than unity, i.e. the embryo is growing



Fig. 9.—Growth of the embryo in relation to that of the ovule in the seeds of Hawkesbury Wonder beans. In the two largest seeds (7 and 9 mm long respectively) one cotyledon has been removed to show the embryonic axis.

relatively faster than the whole seed. After the lag phase the allometric coefficient is very nearly unity, i.e. the embryo and seed are growing at relatively the same rate. By determining the value of the first allometric coefficient genetic differences between varieties would determine the time of onset of the lag phase in relation to seed growth.

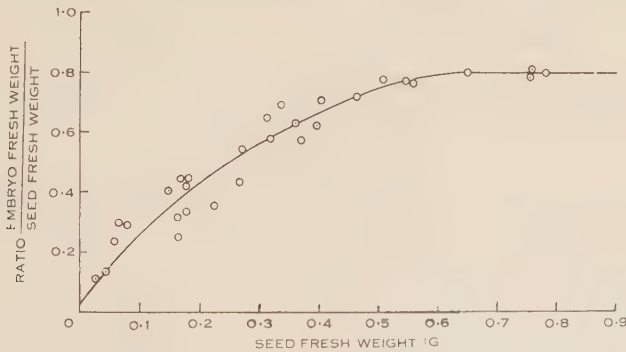


Fig. 10.—Ratio of embryo fresh weight to seed fresh weight plotted against seed fresh weight in a small-seeded variety of peas.

Thus it appears that the onset of the lag phase in pea and bean seed growth is morphologically the result of the embryo having filled the space available within the embryo sac, with the consequence that further embryo growth is limited by the ability of the ovule wall to expand. In beans the lag period begins at about 20 days after anthesis, when the seed is about 9–11 mm long. It is interesting to find that the exponential rate of growth of *Datura* embryos “begins to regress when the embryo starts to bend” and that this bending is associated by Rietsema *et al.* (1955) with the

restriction imposed by limitation of space in the embryo sac. It seems probable that the question which Rietsema and his colleagues pose—is it possible that the limitation of space also causes a slowing down of the growth rate?—must be answered in the affirmative, since it has been shown to fit also the case of bean and pea seeds. The lag period in soybean, clover, and pecan seed growth may also be associated with the filling of the embryo sac by the developing embryo, and may be a phenomenon common to very many seeds. If the case of rye is typical, growth ceases in grass caryopses at a time when the embryo just fills the embryo sac. The embryo sac itself has a diauxic mode of growth, the onset of the lag period coinciding with the cessation of growth of the nucellar pillar (Nutman 1939). Although, according to the data of Rietsema *et al.* (1955), the embryo of *Datura stramonium* does not have a diauxic growth curve, there is in the dry weight increase of the whole seed a lag period which

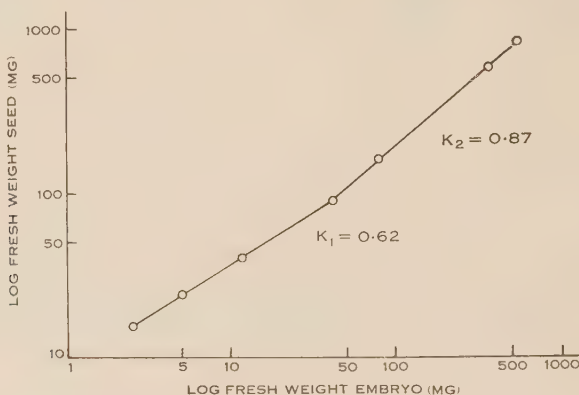


Fig 11.—Hawkesbury Wonder beans: allometry of seed (log fresh weight) and embryo growth (log fresh weight). K_1 , K_2 , allometric coefficients derived from the slopes of the two intersecting lines.

commences at the end of the exponential phase of embryo growth, i.e. when the embryo begins to bend in the embryo sac, and lasts for about 4 days. There may be a causal connection between the sudden decline in growth rate of the embryo and the sudden and coincident decline in dry weight increment of the ovule. Whereas, resumption of growth of the embryo after the lag period is permitted in beans by enlargement of the seed coat, the endosperm having then disappeared, in *Datura* the endosperm may constitute a barrier to a resumption of growth of the embryo. The subsequent changes in dry weight of the *Datura* seed are due to rapid growth of the endosperm, which is virtually compensated for by a loss in dry weight of the seed coat.

A great deal of work has been carried out on the chemical changes in maturing seeds, particularly in the search for criteria for harvesting. In both peas and beans it is perhaps not fortuitous that, at the stage when the embryo/seed fresh weight ratio has just attained the value at which it levels off and the lag period begins, the optimal stage for picking has just been reached. In Dwarf Telephone peas (Bisson and Jones 1932) the optimum picking stage occurs 1 day before the onset of the lag phase. The sugar content of the pea seed falls markedly on the onset of the lag phase (Fig. 6)

and at the same time the pod fresh weight begins to decline as the pods begin to dry out. It is clear that during the lag period a considerable revision of the pattern of metabolism of the seeds must take place, so that when growth is resumed all those changes which are associated with the onset of maturity begin. During this final phase, growth of bean and pea embryos must depend on continued growth of the seed coat which may be regulated by the hormone production of the embryo itself. The fact that the embryo, which, until the lag period, does not conform in shape to the shape of the ovule begins to do so after that period (Fig. 9) suggests that the ovule presents a mechanical restriction to further growth of the embryo. Corner (1951) stated that some legumes have "overgrown" seeds, the embryo continuing to grow apparently by reason of the absence of resistance of the seed coat, and the testa of mature seeds of these species (e.g. *Arachis*) lacks structures typical of legume seeds. However, as far as we are aware, no detailed studies have been made of the growth of such overgrown seeds.

V. ACKNOWLEDGMENT

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A QUANTITATIVE STUDY OF THE GIBBERELLIN CONTENT OF SEEDS OF *PHASEOLUS VULGARIS* AT DIFFERENT STAGES IN THEIR DEVELOPMENT

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Summary

Extracts were made, using ethyl acetate, from bean seeds at different stages of development. Chromatograms of the extracts were developed using *n*-butanol-1·5*N* ammonium hydroxide (3 : 1 v/v) and the chromatograms surveyed by two methods of bioassay for gibberellin activity. Activity was found in two zones, zone 1 at R_F 0·3-0·4, zone 2 at R_F 0·7-0·9. The amount of activity in each zone (compared with that of known amounts of gibberellic acid) changes in step with changes in the growth rate of the seeds. The bean seed has a diauxic pattern of growth. During the first phase of high growth rate, gibberellin activity in both zone 1 and zone 2 rises to a maximum. With the onset of the second (lag) phase of low growth rate, activity in both zones falls to a low level, and at the end of the lag phase zone 2 activity becomes negligible. With the onset of the third phase of high growth rate zone 1 activity rises again and reaches a second maximum, coincident with the maximum growth rate of the seed. Both growth and zone 1 activity then decline.

These data have been interpreted in the light of existing knowledge of gibberellin activity in seeds. It is shown (1) that the fall in gibberellin activity during the first phase of growth cannot be attributed solely to the disappearance of the mucilaginous contents of the embryo sac as the embryo enlarges to fill it; (2) there is probably little connection between the time of cessation of cell division in the seed and the changes in gibberellin activity; (3) that the changes in gibberellin content and in growth rate of the seed coincide so closely that it is not possible to establish the direction of the causal relationship.

I. INTRODUCTION

The presence of gibberellins in higher plants has been amply demonstrated in recent years. The level of gibberellin activity in some seeds, both mature (Murakami 1959*b*) and immature (Phinney *et al.* 1957; Murakami 1959*a*), is remarkably high. Many different kinds of seeds, but not all of those examined, have been shown to contain gibberellins. As gibberellins may play an important role in the growth and development of the seed and possibly of the fruit, quantitative developmental studies on the gibberellins of seeds were commenced in this Laboratory. Data on the growth of the seeds of *Phaseolus vulgaris* (French beans) have already been presented (Carr and Skene 1961), and in this paper data on the pattern of changes in gibberellins in these seeds during their development are given.

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Despite the many reports of gibberellin activity in seeds there have been few quantitative studies and only one (Ritzel 1957) in which the pattern of changes in gibberellin activity during seed development has been traced quantitatively. It is necessary to bear in mind that there may be changes in the levels of more than one gibberellin during seed maturation and possibly also in the content of antigibberellins (Corcoran and Phinney 1958). Changes of this kind in the content of auxin and "growth inhibitors" have already been studied in considerable detail (Nitsch 1952; Luckwill 1959), but it has also been pointed out (Luckwill 1959) that "until the occurrence of (the gibberellins) has been studied in relation to seed and fruit development . . . speculations as to the possible role of naturally occurring gibberellins in the control of fruit growth must of necessity be tentative".

II. MATERIALS AND METHODS

(a) *Bioassay Methods*

Two methods of bioassay have been used. The first is essentially the same as that described by McComb and Carr (1958). Dwarf pea plants (cv. Meteor) are grown in perlite under fluorescent lights giving a 12-hr day and after 10 days the interval between the third and fifth nodes, counting the cotyledonary node as node 1, is measured. A 4- μ l droplet of absolute ethanol containing the material to be assayed is placed in the axil of the scale leaf subtending the third node. Tests have shown that this amount of ethanol does not affect the growth response of the plants. Six days after treatment the interval between the third and sixth nodes is measured and the percentage increment in growth in excess of that of control plants expressed logarithmically. The relation between log per cent. increment and log weight of gibberellic acid is linear over the range 0.001–5 μ g, so by comparing the effects of solutions to be assayed with those of solutions of known gibberellic acid content, activity can be expressed in terms of gibberellic acid. On some occasions when solutions of very low activity were to be assayed, the mode of application to the assay plants was modified to obtain increased sensitivity. Dr. N. J. Scully (personal communication) has found that the entry of gibberellic acid into the plant is facilitated by making a needle puncture through the epidermis. This has been found to increase the sensitivity of the dwarf pea assay by a factor which varies up to about 10. Thus, in order to confirm and measure the low order of activity of some solutions, the stems of the pea plants were punctured at the site of application of the droplet. This modification is referred to in this paper as the "sensitized assay". The linearity of the log response/log dose curve using gibberellic acid does not appear to be affected by the modification. On the occasion of each bioassay, the responses to two levels of gibberellic acid, usually 0.01 and 0.1 μ g per plant, were measured and the data used to calibrate the assay to the log response/log dose graph.

The second bioassay method is based on the wheat leaf assay of Radley (1958). In brief, 4-mm leaf segments prepared from wheat seedlings (cv. Olympic) are incubated in 5 ml of test solution for 20 hr, with horizontal shaking, at 20°C. Solutions are buffered to pH 5 with 0.015M phosphate-citric acid. Between levels of 0.001 and 0.1 p.p.m. gibberellic acid under the above conditions, final segment length is proportional to the concentration of gibberellic acid applied, giving the assay some

quantitative value. The response to sucrose is small, and confusion of sugar-induced growth with the response to the gibberellin(s) of zone 1 (see below) can be avoided by using ethyl acetate for gibberellin extraction, and also by chromatographic separation of the extract. Auxin-induced growth is very slight and takes place only at relatively high auxin concentrations (10 p.p.m.). There appears to be no gibberellic acid-auxin synergism in the assay. Advantages of this bioassay over the dwarf pea assay are that it is less cumbersome and requires a considerably smaller amount of plant extract. Its disadvantages are that it is not as strictly quantitative or as specific as the dwarf pea assay; however, the latter disadvantage can be minimized, as described above.

The data of the bioassay have been analysed using the *t*-test and adopting the 5% level of probability to indicate significance.

(b) *Extraction Methods*

(i) *For Dwarf Pea Assay*.—Beans (*P. vulgaris* cv. Hawkesbury Wonder) were grown in soil in the greenhouse. Pods were harvested when the seeds had attained lengths chosen to represent different stages of maturity. These stages have been described by Carr and Skene (1961). After taking samples for dry weight determination, the seeds were stored in ethyl acetate at -15°C until required. Gibberellin extraction was carried out using ethyl acetate, the actual amount used (300–400 ml) depending on the sample size. Seeds were blended in the extractant, which included that in which they had been stored, for several minutes and the mixture was left overnight at 1°C . After separation of the solid residue, the solution was reduced to a small volume *in vacuo* at a temperature not exceeding 30°C . The concentrate was transferred as a straight line to each of several sheets of Whatman No. 3 chromatography paper, which were then developed simultaneously in a descending system of *n*-butanol–1.5*N* ammonium hydroxide (3 : 1 v/v). The solvent front was permitted to advance 30 cm down the paper from the start line, after which the papers were removed from the tanks and dried in a stream of cold air. The dried chromatograms were cut into 10 3-cm wide horizontal strips each of which was eluted with acetone. Eluates from corresponding strips were combined and reduced to dryness, the residues being taken up in 0.5 ml ethanol. These solutions were then assayed simultaneously with at least two levels of gibberellic acid.

(ii) *For Wheat Leaf Assay*.—As small seed samples suffice for the wheat leaf assay, pods developed from flowers tagged at anthesis were sampled at a known age. At each sampling c. 0.5 g seeds was harvested and immediately extracted at 1°C with 10 ml ethyl acetate for 24 hr. The residue was extracted twice in the same way, the three extracts were combined, and the volume reduced *in vacuo* to a few ml. The extract was transferred to a single piece of Whatman No. 3 chromatography paper and developed as described above. The solvent front was allowed to advance 20 cm from the start line. The paper was then dried thoroughly and cut into 20 strips of equal width covering the whole range of R_F values. The activity on these paper strips was assayed directly by putting each strip into buffer solution containing 10 wheat leaf segments. The responses were compared with those given by two levels of gibberellic acid.

III. RESULTS

(a) Dwarf Pea Assay

Bean seeds were sorted into groups representing five stages of maturity, ranging from about 14 days after anthesis up to about 25 days after anthesis. This period covers most of the first, the whole of the second, and the beginning of the third phase of growth of the bean seed (Carr and Skene 1961). Data on the seed size, seeds per sample, and sample fresh weight are given in Table 1.

The results of the bioassay of the 10 zones of each sample are summarized in Figure 1, in which percentage growth increment in excess of the controls is plotted against R_F value. The histograms marked with an asterisk are those in which the increases in internode lengths of the treated plants were significantly different from those of the controls. There were present two zones of gibberellin activity, the first (zone 1) with a peak at about R_F 0.3–0.4, the second (zone 2) at R_F 0.7–0.9. It is

TABLE 1
SIZES AND WEIGHTS OF SEEDS AT EACH SAMPLING INTERVAL

Sample No.	Mean Seed Length (mm)	Mean Seed Weight (mg)	No. of Seeds per Sample	Fresh Weight of Sample (g)
I	5	11	2241	24.65
II	7	45	823	37.38
III	10	113	344	38.78
IV	13	277	186	51.64
V	15	438	120	52.60

likely that the gibberellin activity of zone 1 is attributable to the presence of gibberellin A_1 . Both gibberellic acid and gibberellin A_1 move at the same R_F as zone 1 in the solvent system used in our experiments, and MacMillan and Suter (1958) have isolated gibberellin A_1 from immature seeds of *P. multiflorus* L. Phinney and Neely (1958) have also identified their "bean factor I", isolated from French bean seed, as gibberellin A_1 , but West and Phinney (1959) have since reported that a positive identification has not yet been made. Zone 2 activity is at the same R_F as the activity detected by McComb and Carr (1958) in Telephone pea plants and by McComb (1959) in germinating Telephone pea seeds. It does not appear to be identical with "bean factor II" which, according to West and Phinney (1959), moves with an R_F of 0.5 in the solvent system we have used. According to Lang (1960), bean factor II is probably identical with the gibberellin A_5 isolated by MacMillan, Seaton, and Suter (1959) from *P. multiflorus* seed. As we have not had the opportunity to compare the behaviour of either bean factor II or gibberellin A_5 with the activity on our chromatograms, it must remain open for the present whether such compounds are present in

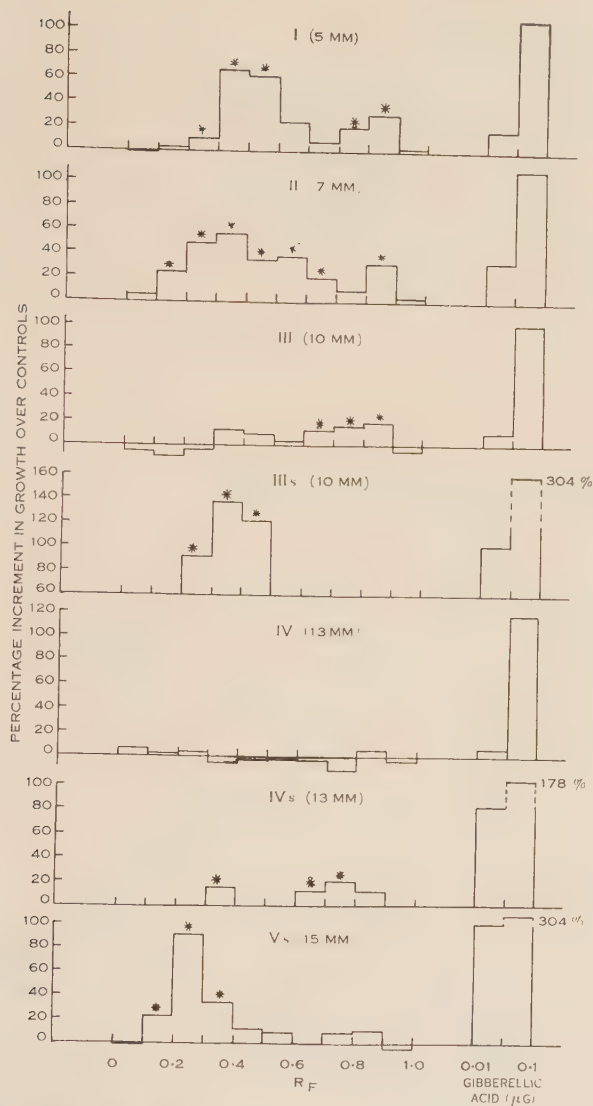


Fig. 1.—Showing gibberellin activities, as measured by responses in the dwarf pea assay, plotted against R_F of the chromatograms of extracts of French beans at different stages of maturity (I-V, see text). The values in parenthesis are the mean lengths of the seeds assayed. Histograms marked with an asterisk are those in which the response is significantly different ($P = 0.05$ or greater) from that of controls. For stages III (10-mm seeds) and IV (13-mm seeds) only, eluates at the R_F values shown in the second diagram were re-assayed using the sensitized assay(s) to confirm activity. However, the levels of zone I activity (see text) in 10- and 13-mm seeds have also been confirmed on further seed samples. The histograms at the right-hand side represent responses to the stated amounts of gibberellic acid per plant. By the method of chromatography used, gibberellic acid would run to R_F c. 0.3.

our extracts. However, it does seem probable that there are at least two substances with gibberellin activity present.

Undoubtedly, other substances with hormone activity are present on the chromatograms. Eluates from the region R_F 0.7–0.9 showed the unusual property of causing growth of the bud in the leaf axil to which they were applied. Such buds continued to grow until they were about 1.5 cm long. This did not occur in either control plants or those treated with gibberellic acid; nor could it be induced experimentally by applying kinetin to the leaf axil. Whether or not this "bud growth factor" is identical with the gibberellin of zone 2 remains to be investigated.

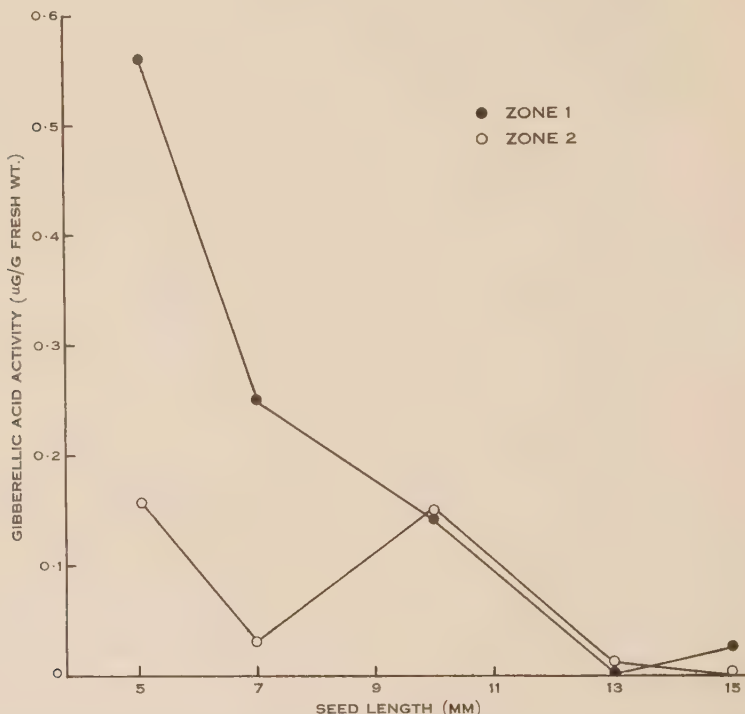


Fig. 2.—Gibberellin activity (μg gibberellic acid) per gram fresh weight of seeds plotted against seed length. Zone 1 refers to activity on the chromatograms in the region R_F 0.3–0.4, zone 2 to activity in the region R_F 0.7–0.9. The seed length is a measure of the stage of development of the bean seed (see Fig. 4). Data from dwarf pea bioassay.

The pattern of change in the concentration of the gibberellins of zones 1 and 2 during the development of the seeds is shown in Figure 2. Activity is plotted as μg gibberellin (as gibberellic acid) per g fresh weight against seed length, representing stages of maturity. It should be remembered that the activities, weight for weight, of zones 1 and 2 are not necessarily identical, using gibberellic acid as a common basis for comparison. In fact, it is unlikely that the substances of zone 1 and zone 2 would have equal growth-promoting activity. Furthermore, there is evidence that the growth-promoting effects of zone 1 are longer lasting in the pea plant than are those

of gibberellic acid. In one experiment, for instance, most of the growth acceleration induced by gibberellic acid occurred before the date at which the routine bioassay measurements were taken, while the growth acceleration induced by treatment with zone 1 eluate continued for at least a further 5 days. This resulted in a significant diminution in the difference in height between the group of plants treated with zone 1 eluate and the group treated with $0.1 \mu\text{g}$ of gibberellic acid (Table 2). Radley (1958) had also noted the same phenomenon.

Referring again to Figure 2, it is evident that zone 1 activity is very high in the very immature seed ($0.56 \mu\text{g/g}$ fresh wt. in seeds 5 mm long) and falls quite rapidly until at the end of the lag phase in seed growth it reaches its lowest level ($0.0024 \mu\text{g/g}$ in 13-mm seeds). On the other hand, zone 2 activity, on a fresh weight basis, although initially lower than that of zone 1, remains fairly steady during the

TABLE 2
HEIGHT INCREMENT OF METEOR PEA SEEDLINGS IN PERIODS
SUBSEQUENT TO APPLICATION OF ZONE 1 ELUATE AND OF
GIBBERELLIC ACID

Time from Application (days)	Increment in Height over Controls (%)	
	Zone 1 Eluate	$0.1 \mu\text{g}$ Gibberellic Acid per Plant
6	61.9	109.0
11	74.3	81.3

first exponential phase of seed growth, but it too falls to a low level ($0.0047 \mu\text{g/g}$) in the 13-mm seed. Even the sensitized assay is too insensitive to measure the still lower level of zone 2 activity in 15-mm seeds, which just fails to attain statistical significance. After the lag phase, zone 1 activity increases again, although in 15-mm seeds it is still relatively low ($0.023 \mu\text{g/g}$).

The data are plotted as activity (in terms of gibberellic acid) per 1000 seeds against seed length in Figure 3. On a per seed basis, the gibberellin activity in both zones 1 and 2 rises during the first phase of seed growth and reaches its maximum just before the onset of the lag phase in growth of the seed. When activity per seed is at its maximum (in seeds about 9–10 mm long) the activity is about equally distributed in zones 1 and 2. During the lag phase there is a marked fall in activity per seed. In 13-mm seeds, zone 2 activity per seed is approximately at the same level as zone 1 activity but with the resumption of active growth following the lag phase the zone 2 activity declines still further and is inappreciable in 15-mm seeds, while the zone 1 activity increases concomitantly with the increase in the growth rate.

(b) Wheat Leaf Assay

One of the difficulties with the dwarf pea assay is the relatively large quantity of plant extract required. Using this assay, it was necessary to make mass collections of beans and to divide the collections into size classes, representing different stages of maturity. With the wheat leaf assay, on the other hand, the numbers of seeds required for extraction was relatively small. This meant that flowers could be tagged on the day of anthesis and the pods which developed used for both growth studies and gibberellin assays. As the wheat leaf assay appears to be not very sensitive to the

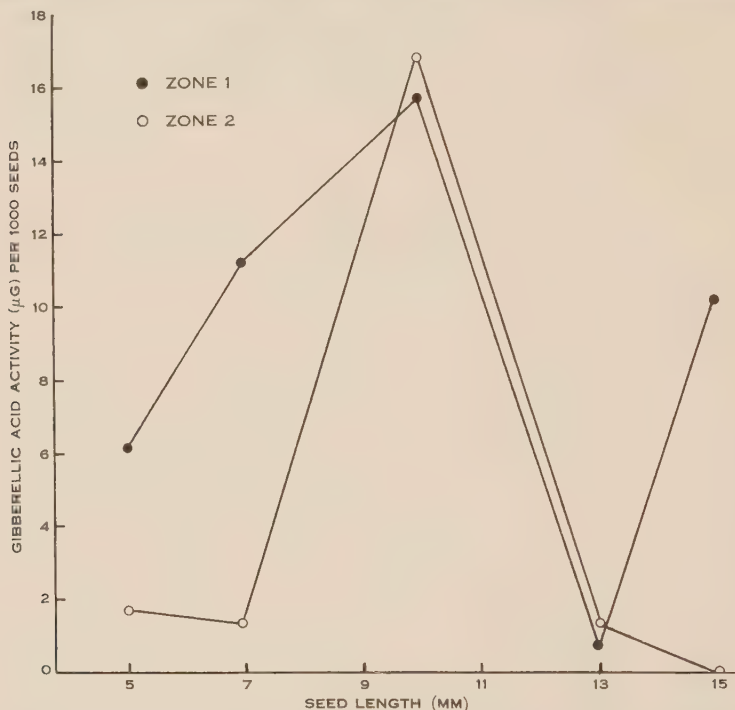


Fig. 3.—Gibberellin activity (μg gibberellic acid) per 1000 seeds, plotted against seed length. The lag phase in seed growth begins when the seed is about 9–10 mm long. Data from dwarf pea bioassay.

gibberellin of zone 2, only the activity of zone 1 has been followed by means of this assay. Its use, however, has enabled us to fill in a considerable amount of the detail of the pattern of changes in gibberellin activity, particularly in very young seeds (of low weight but high activity) and almost mature seeds (of high weight but low activity). We agree with Lang (1960) that it is essential to assay extracts containing natural gibberellins on more than one type of test plant and it is encouraging that the pattern of changes revealed by the wheat leaf assay (Fig. 4) is both qualitatively and quantitatively in full agreement with that revealed by the dwarf pea bioassay.

The activity per seed is shown to fall rapidly during the later stages of the first phase of seed growth, conforming to the pattern shown in Figure 3, having risen during the earlier, exponential stage. Zone 1 activity remains low during the lag

phase then rises very rapidly as exponential growth begins again at the onset of the third phase of growth. The maximum level per seed is attained at 26 days after flowering when the seeds are 18–20 mm long. After this, zone 1 activity declines rather slowly until, in the mature dry seed, little or no activity can be detected by the wheat leaf assay. There are changes in the distribution of activity along the chromatogram during the later stages of seed maturation, with the appearance of new zones of activity, but these have not yet been investigated in detail.

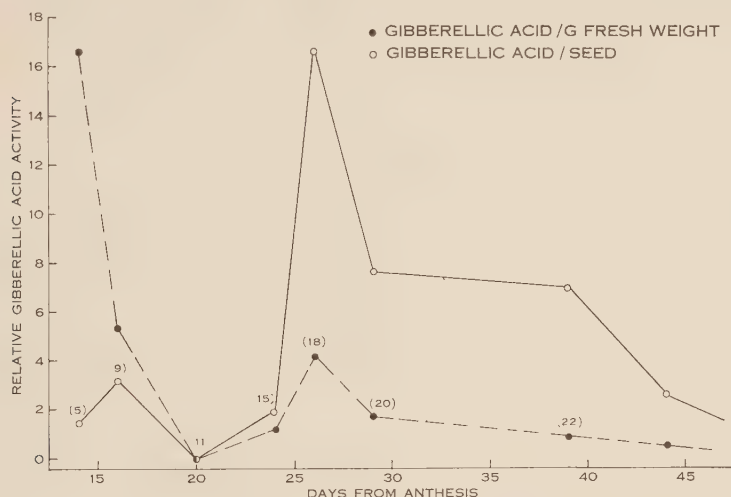


Fig. 4.—Gibberellin activity (relative values) per gram fresh weight and per seed plotted against days from anthesis. These data were obtained by the wheat leaf segment bioassay. Values in parenthesis indicate seed lengths (mm) at the corresponding number of days from anthesis.

IV. DISCUSSION

Growth studies of French beans (Carr and Skene 1961) have shown that the seed growth takes place in three phases. The seeds begin to grow about 9 days after anthesis and growth is exponential up to about the 17th or 18th day and then begins to slow down. During this first period, the embryo grows relatively faster than the seed. When the embryo just fills the embryo sac, growth of the seed slows down and enters a lag phase. This lasts from about the 20th to the 23rd day, after which the seed commences to grow rapidly again up to about the 26th day after anthesis and then growth gradually declines, eventually to zero.

There are thus two phases of rapid growth separated by a brief phase of very slow growth. The gibberellin activity correlates remarkably well with this diauxic pattern of seed growth. The gibberellin activity per seed in zone 1 has been plotted together with the increment curve of fresh weight per 3 days in Figure 5. It is apparent that when the seeds are growing rapidly the gibberellin content is high; when growth is declining the gibberellin content is falling. This synchronization of gibberellin activity with growth rate is so close that it raises the problem of which is cause and which effect.

On closer analysis it becomes clear that the situation is much more complex than the comparisons in Figure 5 would suggest. The end of the first phase of growth coincides with the filling-out of the embryo sac by the seed and the disappearance of the last remnants of the "non-cellular endosperm" (or mucilaginous contents of the embryo sac). Since the endosperm of some seeds, e.g. *Echinocystis* (Phinney *et al.* 1957), is known to be a rich source of gibberellin activity it might be suggested that the fall in gibberellin activity of the whole seed at the end of phase 1 is due merely to the disappearance of the mucilaginous contents of the embryo sac. However, there is some evidence that this cannot account entirely for the changes in level of activity

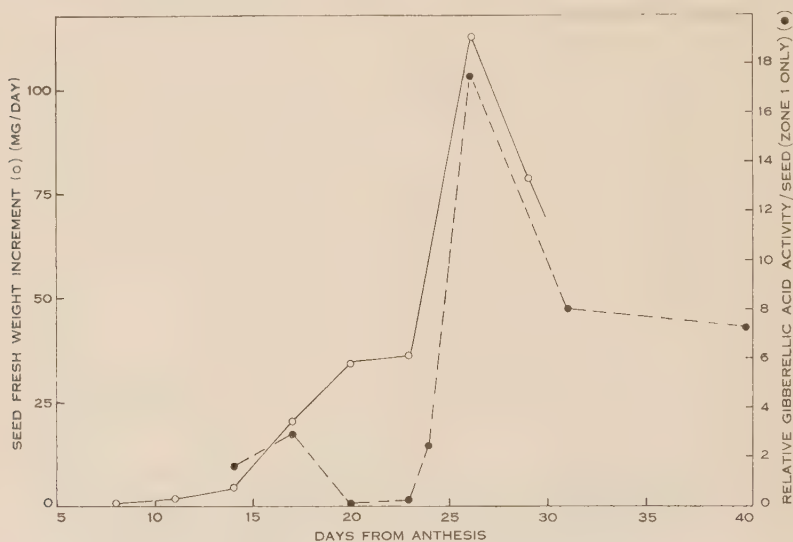


Fig. 5.—Increment in fresh weight (mg/day) and relative gibberellin activity per seed, plotted against days from anthesis, to show the close correspondence between the growth rate and gibberellin activity in developing bean seeds. The data for gibberellin activity are for zone 1 only, and where they are available the data from the dwarf pea bioassay have been given preference over those from the wheat leaf bioassay. Nevertheless, the data from the two methods of bioassay are not noticeably discordant (see Figs. 2, 3, and 4).

in the first phase of growth. For instance, supposing that in the above case the concentration of gibberellin in the embryo sac mucilage remained constant, the amount of gibberellin activity in the embryo sac would increase only up to the stage when the seeds are about 5 mm long, because after that stage the growth in volume of the embryo is greater than the growth in volume of the embryo sac (cf. Carr and Skene 1961, Fig. 9). The data of Figure 3 show that activity in both zone 1 and zone 2 is increasing even in seeds 7 mm long, that is, when the contents of the embryo sac are decreasing in volume. Also, examination of the gibberellin content of the immature embryos excised from the seeds has provided evidence that a considerable fraction of the activity in the seed before the onset of the lag phase in growth is in the embryo, although the contents of the embryo sac appear to contain activity as well (Skene, unpublished data). Radley (1958) claims that gibberellin activity in immature

(2–6 mm long) runner bean seeds is approximately equally distributed between the “embryos”, cotyledons, and testas, on the basis of the extracted weights of these parts. It is difficult, however, to obtain very small embryos free from all traces of the mucilaginous contents of the embryo sac and it would be preferable to assay these contents separately.

Although the fall in zone 2 activity may be connected with the disappearance of the “endosperm” towards the end of the first phase of growth, the fact that the zone 1 activity rises to a high level in the third phase of growth shows that the presence of the endosperm is at least not essential for continued gibberellin production, and could indicate that the endosperm is not the sole source of activity during the first phase of growth. Moreover, the fact that a single zone of activity, at the same R_f as our zone 2, was found by McComb (1959) in extracts of imbibed mature pea seeds of a tall variety and in extracts of growing plants of the same variety (McComb and Carr 1958) is evidence that the gibberellin(s) of zone 2 are not formed exclusively, if at all, in the endosperm.

The slowing down of growth during the lag phase presents an interesting problem, since it is contemporaneous with (1) the filling of the embryo sac and (2) a rapid fall in gibberellin activity. At least two hypotheses may be put forward to account for the coincidence of these phenomena. According to the first, the mechanical hindrance imposed by the testa might slow down the growth of the embryo and the slowing down might result in metabolic changes, including the production of less extractable gibberellin. This would imply that gibberellin production would be an effect rather than a cause of seed growth. As a corollary to this, some form of plasticization of the testa might be set in train during the lag phase, allowing growth and gibberellin production to proceed during the third phase. The second hypothesis would state that since very many changes in the metabolic and biochemical activities of the seed and fruit appear to coincide with the onset of the lag phase, the fall in growth rate of the whole seed and its parts might be the resultant of a drastic revision of the pattern of metabolism of the seed, one aspect of which could be a reduced output of gibberellins. On this viewpoint the fall in growth rate could be an effect of the reduced content of extractable gibberellin, i.e. growth might be regulated by the level of gibberellin activity. This hypothesis would involve the premise that it is either the extractable gibberellin itself which is effective in regulating growth, or that this is directly proportional to that fraction of the total gibberellin content which is effective. On a per seed basis, the activity of both zones 1 and 2 rises during the first exponential phase of growth and reach a maximum at the end of it. It can be ascertained from the work of McKee, Robertson, and Lee (1955) on peas that cell number in the seed ceases to increase long before the end of the exponential phase of dry weight increase. If this is also true of beans, there is likely to be little correlation between the amount of activity of either zone 1 or zone 2 and the intensity of cell division in the developing bean seed.

A considerable interest attaches to the question of the locus of synthesis of the gibberellins of the seed. According to Radley (1958), all parts of the bean seed—the cotyledons, “embryo”, and testa—contain approximately equal amounts of gibberellin activity, on a basis of the weights of these parts after extraction. Since the

total weight after extraction was about half of the fresh weight of the seeds before extraction, it is difficult to estimate whether Radley's statement can be applied to the fresh weights of the parts before extraction. In any case, from a consideration of the data of Figures 2 and 3 it seems unlikely that the gibberellin activity of French bean seeds is uniformly distributed in all parts of the seed and it certainly does not remain a constant fraction of the fresh weight of the seed. The fact that the activity per seed is rising during the first phase of growth while the activity per gram fresh weight is declining rules out the possibility that the seed commences growing with a fixed "capital" of gibberellin which is merely diluted by increase in size of the seed. There must be further production of gibberellin during growth; it is more probable that only a limited number of production loci are involved and that these constitute a diminishing fraction of the total weight than that the decline in gibberellin per gram fresh weight is due to a uniform dilution throughout the mass of the seed. The embryonic axis (embryo minus cotyledons) probably contains gibberellin (Radley 1958) and the rapid rise and eventual fall in the growth rate of the embryonic axis during the third phase of seed growth correspond very closely in time with the second rise and eventual decline in zone 1 activity. However, the embryonic axis is not the only part of the seed which is growing rapidly during the early part of phase 3 and on a per seed basis much, if not all, of the gibberellin activity in 20-mm seeds is in the cotyledons (Skene, unpublished data).

It is of interest to compare the level of gibberellin activity of French bean seeds with the data of the many published reports of gibberellin activity in seeds. However, in the majority of these the level of activity is not stated quantitatively. In lupins and beans, to quote Ritzel (1957), "both activity per seed and activity per gram of seed increased with seed age and there was evidence of loss of activity only after the seed approached final size". No values are given by Ritzel, but Murakami (1959*b*) states that the concentration of gibberellins in mature, dry *Lupinus luteus* seeds is approximately equivalent to $0.1 \mu\text{g/g}$ of "gibberellin A". As he points out, this is a relatively high value and it may be in accord with Ritzel's data. Radley (1958) states that the high level of activity in immature runner bean seeds falls in the mature seed to a level comparable with that in the growing plant. In seeds 2–6 mm long, activity was equivalent to $0.25 \mu\text{g}$ of gibberellic acid per gram fresh weight of seed. This agrees well with the range $0.25\text{--}0.56 \mu\text{g/g}$ fresh weight reported above for zone 1 activity. In none of these cases have the changes in activity demonstrated above been recorded, but it is particularly interesting to recall that Mitchell, Skaggs, and Anderson (1951) found that the amount of ether-extractable hormones increased in Black Valentine bean seeds up to about 8 days after pollination and then decreased rapidly to an immeasurable amount (when assayed on the same variety of bean plant) on about the 15th day. These early data are in close accordance with the pattern of changes in gibberellin content of French bean seed during the first and second growth phases.

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IONIC RELATIONS OF CELLS OF *CHARA AUSTRALIS* R. BR.

IV. MEMBRANE POTENTIAL DIFFERENCES AND RESISTANCES

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Summary

Experiments are described in which the electric potential difference and resistance between the cytoplasm and the external medium were measured in cells of *Chara australis*. The method was designed to eliminate the effect of the negatively charged Donnan system of the cell wall. Both the potential difference and the resistance are attributed to the outer cytoplasmic membrane. It is shown that they may be quantitatively explained by the passive diffusion of potassium and sodium ions across the membrane with permeabilities of the order of 10^{-5} and 10^{-6} cm sec⁻¹ respectively. The resistance-voltage characteristic of the membrane is accurately predicted by the constant field equation of Goldman (1943). The significance of these findings is discussed.

I. INTRODUCTION

The resting potential of single cells of the Characeae has been the subject of much investigation. The resting potential is the electric potential difference (p.d.) between the vacuolar sap of the cell and the medium bathing the cell. It is best measured by means of salt-bridges in contact with these phases. However, in much early work (e.g. Osterhout and Harris 1929; Osterhout 1930; Osterhout and Hill 1938) measurements were made of the p.d. between two different liquid contacts on the cell surface. Microelectrodes inserted into the cell were employed by Umrath (1930, 1934), Studener (1947), and Walker (1955). It was shown that, in *Nitella*, the resting potential arises as a p.d. between the external medium and the cytoplasm, there being no measurable p.d. between the cytoplasm and the vacuolar sap (Walker 1955). The effects of the concentrations of ions in the external medium have been often investigated; increasing concentrations of cations reduce the magnitude of the resting potential, potassium having a greater effect than other cations. Only Osterhout has attempted a quantitative treatment.

The resting potential of the cell has been ascribed to various mechanisms, among them being:

- (1) A diffusion potential in one or more cellular membranes which separate phases of different ion concentrations (see Osterhout 1952);
- (2) A redox potential in a layer (membrane) allowing electronic conduction and separating phases of different redox potentials (see Lund 1947);
- (3) A membrane containing oriented dipoles (Umrath 1942); and

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- (4) A Donnan distribution due to the presence of indiffusible ions in the cytoplasm of the cell (Blinks 1940; Hope and Robertson 1953).

Blinks and Pickett (1940) have provided experimental evidence against the redox mechanism (2), and there has been no theoretical or experimental justification advanced for the dipole mechanism (3). The Donnan mechanism (4) seems unable, alone, to account for the different effects of sodium and potassium ions on the resting potential; it may, however, occur in combination with another mechanism such as (1).

Osterhout proposed mechanism (1), and supported it by many experimental studies. There are a number of reasons for re-examining the question. His results were compared with the predictions of Henderson's equation for liquid junction potentials, an equation which is simple, but which is based on an impossible assumption (Johnson, Eyring, and Polissar 1954). His experiments, too, are sometimes of doubtful validity—e.g. the permeability ratio for potassium to sodium was calculated from potentials measured in 10 mN KCl. This concentration of KCl, as Osterhout himself showed, frequently causes an abnormal change in resting potential, which is related to the action potential.

The measurements described in this paper were made with inserted micro-electrodes, and gave the p.d. between medium and cytoplasm or medium and vacuole directly. These p.d.'s. are here assumed to be equal. Methods using external contacts, such as that of Osterhout, give only the algebraic sum of two such p.d.'s. The present experiments were designed to keep the Donnan potential of the cell wall constant, while keeping the cell under nearly physiological conditions. The Donnan potential of the wall was not involved in the overall p.d. between medium and cytoplasm. The measured p.d.'s are compared with the predictions of modern equations for diffusion potentials across membranes.

The first measurements of membrane resistance in these cells were made by Blinks (1930, 1936), using direct current (d.c.). The highest surface specific resistance he found, 250 k Ω cm²,* has been widely quoted (Cole 1942; MacRobbie and Dainty 1958) without consideration of the conditions of his experiment. He observed that potassium ions in the external medium reduced the resistance more than sodium, lithium, and other ions. Neither he, nor Umrath (1940), nor Weidmann (1949) offered a quantitative interpretation of their results. Bennett and Rideal (1954) made measurements of resistance in *Nitella* cells using a microelectrode of Ag/AgCl, but since they used alternating current of frequency 1000 c/s the current flow was not homogeneous through the cell surface. Thus their measured resistances and capacitances are difficult to interpret. In none of these experiments is it possible to distinguish the contributions of the tonoplast and plasmalemma to the measured resistance. Walker (1957, 1960) found in *Nitella* that the plasmalemma contributed most or all of the measured membrane resistance. The values of surface specific resistance found by different workers have varied widely between the 250 k Ω cm² of Blinks (1930), the 50 k Ω cm² of Findlay (1959), and the 5 k Ω cm² of Walker (1960).

The present paper reports measurements of membrane resistance as a function of the composition of the external medium and as a function of current density.

* i.e. 250,000 ohms for each cm² of cell surface.

These were made concurrently with the measurements of membrane potential. A quantitative treatment of these sets of data is attempted, in terms of the permeabilities to ions of the plasmalemma, and of the ionic activities on each side of this membrane.

II. EXPERIMENTAL METHOD

The material used was *Chara australis* R.Br. var. *nobilis* A.Br.; it was collected from field ponds and remained viable for some weeks in the laboratory in an artificial pond water.* Cells for experiment were freed from neighbouring cells, and soaked for 2–6 hr, or occasionally overnight (16 hr) in 5.0 mN NaCl solution, and then transferred to 1.0 mN NaCl+0.1 mN KCl to await use. The aim was to remove the exchangeable calcium from the cell wall. During experiments the cells were bathed in a flow of fresh solution of constant normality (1.1 mN or 2.0 mN total). The effects of changes in the ratio of sodium to potassium or in the nature of the anion were measured.

Measurements of membrane potential and resistance were made with two inserted microelectrodes; the method was similar to that of Walker (1960). Two reference electrodes dipped into the external solution; a calomel half-cell and salt-bridge for potential measurements, and a coiled Ag/AgCl electrode for resistance measurements. The microelectrodes were generally inserted into the vacuole a distance of 100–200 μ . Under these conditions the “seal” described by Walker (1955) between the glass of the electrode and the cytoplasm did not form within 6 hr. After the insertion of the electrodes the potential was frequently steady (± 0.5 mV) within 2 min.

The electrical measuring apparatus was similar to that used by Walker (1960). It was capable of measuring p.d. to within ± 0.1 mV, but usually the accuracy was ± 0.5 mV. Current was measured to within $\pm 2\%$. The measurement of membrane resistance was accurate to about $\pm 5\%$.

III. RESULTS

(a) Potential Difference Measurements

The p.d. between cytoplasm and medium during two types of change in the external medium is shown in Figure 1. In one experiment (Fig. 1(a)), the external medium was changed from 0.1 mN KCl to 1.0 mN KCl; in the other (Fig. 1(b)), it was changed from 1.0 mN NaCl+0.1 mN KCl to 0.6 mN NaCl+0.5 mN KCl. It would be expected that in the latter case (but not in the former) the Donnan potential of the cell wall would remain constant. Comparison of the time courses in Figure 1 shows a marked difference. The rapid initial rise in curve (a) is absent in (b), which suggests that it is a change in the wall potential. The monotonic rise in (b) is due to a membrane which distinguishes between potassium and sodium; presumably the plasmalemma. The rise in potential has a half-time of 3–5 min, the significance of which is discussed below.

* This contained Na, 1.0 mN; Ca, 0.5 mN; K, 0.1 mN; and Cl, 1.6 mN.

In the following experiments the p.d. was measured at equilibrium after a change in the concentration of potassium (and sodium), *keeping the total concentration constant* as in Figure 1(b). On restoring an original concentration after such a change, the p.d. generally returned to a value within 2–4 mV of the original. A typical sequence of external concentrations of potassium chloride (K_o) (with that of sodium (Na_o) in parenthesis) was 0.1 (1.0), 0.25 (0.85), 0.4 (0.7), 0.7 (0.4), 1.0 (0.1), 0.7 (0.4), 0.4 (0.7), 0.25 (0.85), 0.1 (1.0) mN. In such experiments, when the repeatability was as good as mentioned above, the mean of the two determinations of p.d. (E_{oi})* for a given K_o was plotted against $\log_{10} K_o$. Figure 2 shows a typical

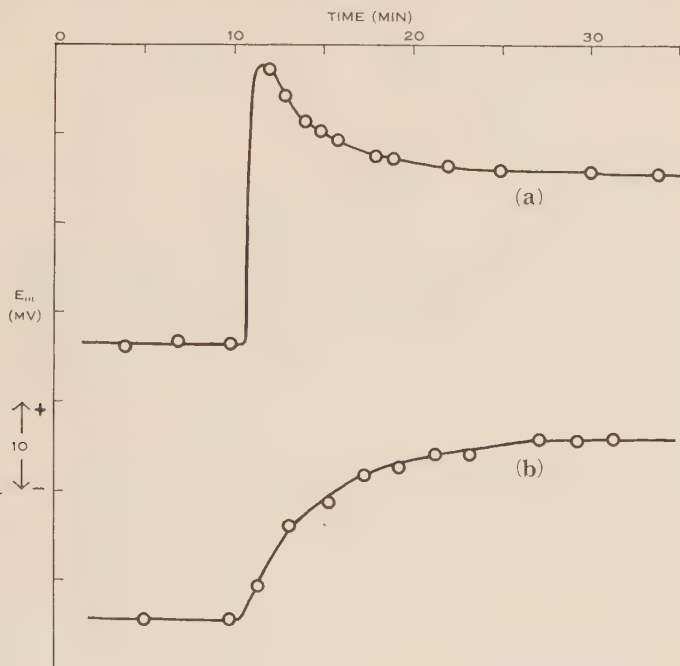


Fig. 1.—Time courses of the potential difference between the interior of the cell and the bathing medium (E_{oi}). In each case the potassium concentration in the bathing medium was changed at 10 min on the time scale. (a) Bathing medium changed from 0.1 mN KCl to 1.0 mN KCl. (b) Bathing medium changed from 0.1 mN KCl+1.0 mN NaCl to 0.5 mN KCl+0.6 mN NaCl.

result where the external anion was chloride, and the total external cation concentration was 1.1 mN. Similar results were obtained with anions other than chloride, and with a total cation concentration of 2.0 mN (Fig. 3). At higher total cation concentrations, the high potassium end of the range could not be explored, as spontaneous action potentials occurred. In each of Figures 2, 3, and 4 a theoretical curve has been drawn, based on the equation:

$$E_{oi} = (RT/F) \ln[(K_o + \alpha \cdot Na_o)/(K_i + \alpha \cdot Na_i)], \quad (1)$$

where α ($= P_{Na}/P_K$) is the ratio of the permeabilities of the interface between o and

* $E_{oi} = E_i - E_o$, and thus has the sign of phase i relative to o .

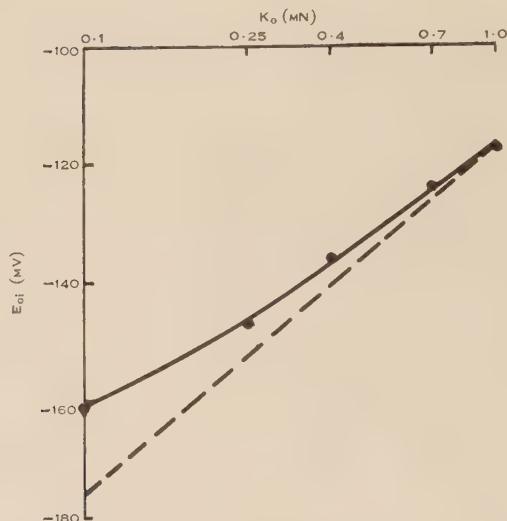


Fig. 2.—Potential difference between the cell interior and the bathing medium, as a function of the potassium concentration in the medium (keeping $K_o + Na_o = 1.1$ mN). ● Experimental points (means for one cell). — Fitted curve based on equation (1), with $a = 0.074$ and $K_i + a \cdot Na_i = 97$ mN. - - - Limiting slope of 58 mV per log unit ($a = 0$).

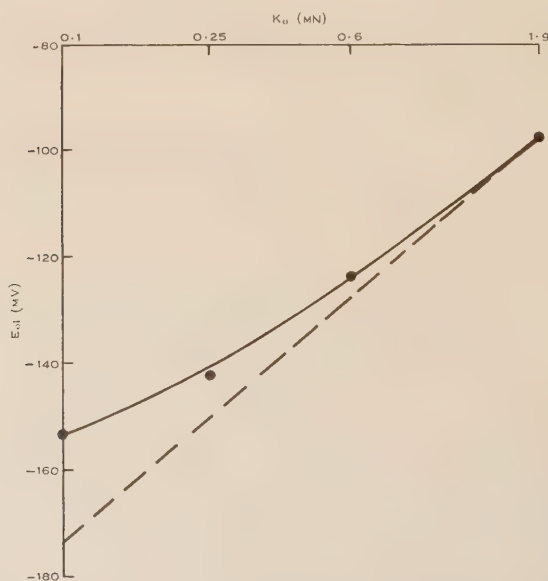


Fig. 3.—Potential difference between the cell interior and the bathing medium, as a function of the potassium concentration in the medium (keeping $K_o + Na_o = 2.0$ mN). ● Experimental points (means for one cell). — Fitted curve based on equation (1), with $a = 0.059$ and $K_i + a \cdot Na_i = 90$ mN. - - - Limiting slope of 58 mV per log unit ($a = 0$).

i to sodium and potassium, and $(K_i + a \cdot Na_i)$ is an internal concentration parameter. Both a and $(K_i + a \cdot Na_i)$ are assumed constant (see Section IV).

Although the behaviour depicted in Figures 2 and 3 was frequently obtained, in some experiments consistent differences between such theoretical curves and the experimental points were noted. Figure 4(a) illustrates deviations from the "normal" curve at the high potassium end of the concentration range and Figure 4(b) at the high sodium end.

One of the assumptions implicit in equation (1) is that the membrane is not significantly permeable to the anion. In some experiments the anion was changed

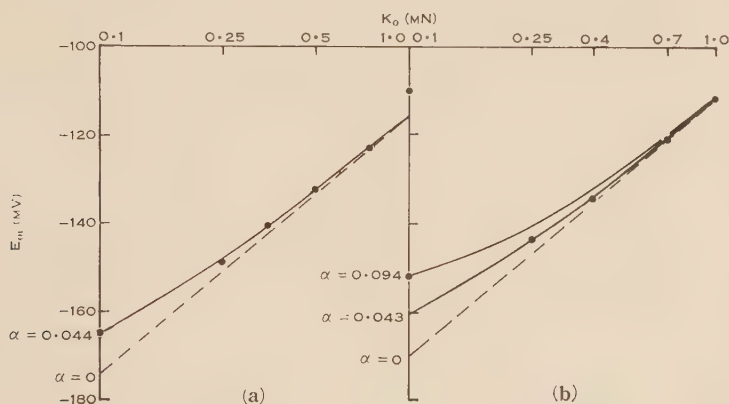


Fig. 4.—Potential difference between the cell interior and the bathing medium as a function of the potassium concentration in the medium ($K_o + Na_o = 1.1$ mN). (a) and (b) are different experiments. ● Experimental points (means for one cell). — Fitted curves based on equation (1): values of a used in the calculation are given. - - - Limiting slope of 58 mV per log unit ($a = 0$).

from chloride to sulphate, nitrate, benzenesulphonate, or glucuronate. A very quick change in p.d. occurred, suggesting a diffusion p.d. in the cell wall. With some anions the p.d. for chloride was reverted to, but with others the change in p.d. persisted for up to 15 min which was the maximum duration of the experiment. The steady values of p.d. are given in Table 1.

In addition, the effect of substituting choline for sodium was examined. The p.d. changed always to a more negative value, with a half-time of 1–2 min. Table 2 lists the results of such experiments.

(b) Resistance Measurements

During the experiments already described, measurements were made of the membrane resistance of the cells. In some experiments the resistance was measured as a function of current density for each value of the external potassium concentration. In others, the resistance for very small current flows only (both positive and negative) was measured for each concentration. The values obtained must be corrected for the series resistance of the external medium and of the cell sap (Walker 1960); however, the correction for the resistance of the sap was found to be small, and it was neglected.

The corrected values were then converted to membrane resistances (r , in ohm cm^2). As previously, the resistance used was the gross d.c. resistance, given by:

$$R = (\Delta E_{oi})/I, \quad (2a)$$

where I is the current, and not the incremental d.c. resistance given by

$$R' = \partial E_{oi}/\partial I. \quad (2b)$$

TABLE 1

CHANGES IN POTENTIAL DIFFERENCE ON SUBSTITUTING VARIOUS ANIONS FOR CHLORIDE
Total anion concentration = 1.1 mN: $\text{Na}_o = 1.0$ mN and $\text{K}_o = 0.1$ mN in all experiments except experiment 5, where $\text{K}_o = 1.0$ mN and $\text{Na}_o = 0.1$ mN

Expt. No.	External Medium Change	Potential Difference (mV)		
1	Chloride, sulphate	-151,	-151	
2	Chloride, nitrate, chloride	-147,	-149,	-147
3	Chloride, benzenesulphonate, chloride	-156,	-167,	-159
4	Chloride, benzenesulphonate, chloride	-161,	-169,	-162
5	Chloride, benzenesulphonate, chloride	-117.5,	-127.5,	-116.5
6	Chloride, glucuronate, chloride	-182,	-188,	-180
7	Chloride, glucuronate, chloride	-178,	-185,	-180

A typical set of results on one cell is shown in Figure 5. The resistance was highest in high sodium media and lowest in high potassium, and the rise of resistance with negative current (rectification) was greatest in the media with low potassium.

TABLE 2

CHANGES IN POTENTIAL DIFFERENCE ON SUBSTITUTING CHOLINE FOR SODIUM

External Medium	Potential Difference (mV)				
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5
(i) Potassium 0.1 mN, sodium 1.0 mN	-170	-146	-157	-147.5	-144
(ii) Potassium 0.1 mN, choline 1.0 mN	-183	-160	-164	-159	-157
(iii) Potassium 1.0 mN, sodium 0.1 mN	-127	-108	-108	-107	-117
(iv) (ii)–(iii) (see text, p. 38)	-56	-52	-56	-52	-40

All cells gave qualitatively the same picture, with some variation in the actual resistance from cell to cell. Figure 6 shows the membrane resistance at zero current plotted against K_o for five cells.

Table 3 gives the results of some measurements of resistance in media in which other anions were substituted for chloride. For times of up to 15 min, the resting resistance was not changed by the anion substitutions.

In calculating the membrane resistance from the product of the measured resistance (corrected as mentioned above) and the surface area of the cell, it is assumed that the current flow is homogeneous over the cell surface. This depends on the homogeneity of the cell surface. In addition, if the longitudinal resistance of the vacuolar sap were high enough relative to the membrane resistance, most of the current would leave the cell in the neighbourhood of the implanted current-carrying microelectrode. The membrane resistance calculated using total surface

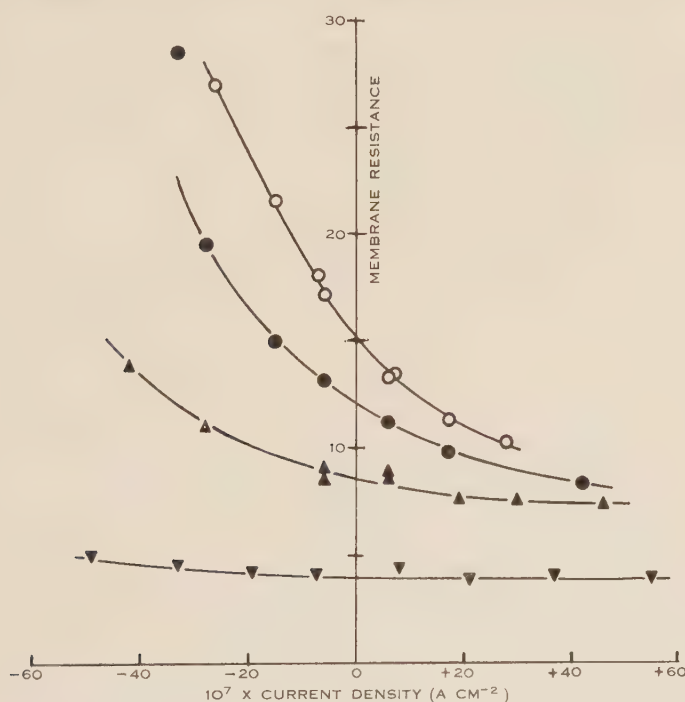


Fig. 5.—Membrane resistance (in $k\Omega\ cm^2$) between the cell interior and the medium as a function of the current density in various bathing media. Curves connect points in one medium, and are *not* calculated from theory. All measurements made on one cell:

\circ $Na_o = 1.9$, $K_o = 0.1\ mN$

\bullet $Na_o = 1.6$, $K_o = 0.4\ mN$

\blacktriangle $Na_o = 1.2$, $K_o = 0.8\ mN$

\blacktriangledown $Na_o = 0.1$, $K_o = 1.9\ mN$

area would then be an overestimate. To confirm that the current flow was nearly homogeneous, the change in potential of a microelectrode just *external* to the cell was measured in response to a small current passed between an implanted microelectrode and the coiled Ag/AgCl electrode lying parallel to the cell, as a function of distance from the current microelectrode. Such a series of measurements is shown in Figure 7, which shows that the current density is nearly independent of the distance from the current-carrying probe. Cells with length greater than 2 cm were seldom used. Very close to the region where the current probe had entered the cell, the current density was sometimes 1.5–2 times the average over the cell length, more particularly immediately after the probe had entered, indicating a local low membrane resistance. Since in the experiment of Figure 7 K_o was 1.0 mN and the membrane

resistance about $5 \text{ k}\Omega \text{ cm}^2$, the current flow would have a greater tendency towards inhomogeneity than with lower concentrations of potassium and higher membrane

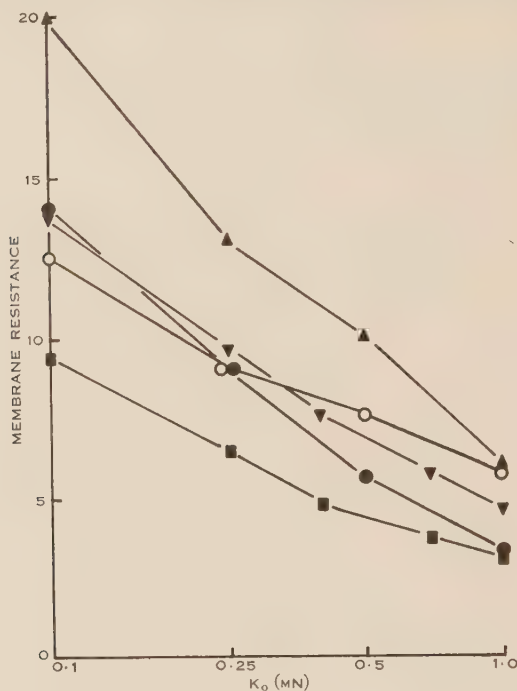


Fig. 6.—Membrane resistance r_o (in $\text{k}\Omega \text{ cm}^2$) at zero current as a function of the potassium concentration in the medium. Curves connect points representing one cell, and are not calculated from theory. ○, ●, ▲, ▼, and ■ represent experiments on different cells.

resistances. Little difference was found in the distribution of current through the cell in media containing 0.1 mN and 1.0 mN KCl. Thus the assumption of homogeneous

TABLE 3
MEMBRANE RESISTANCE AFTER ANION SUBSTITUTIONS
Membrane resistance (r_o) in $\text{k}\Omega \text{ cm}^2$

External media (mN)*	KCl 0.1 NaCl	KCl 0.1 Na glucuronate	KCl 0.1 NaCl	KCl 0.1 $\text{C}_6\text{H}_5\text{SO}_3\text{K}$	KCl 0.1 $\text{C}_6\text{H}_5\text{SO}_3\text{Na}$	KCl 0.1 NaBr	KCl 0.1 NaCl
Cell 1	13.7	13.0	12.0	12.5	—	—	12.0
Cell 2	12.0	12.0	11.7	—	—	—	—
Cell 3	12.5	14.5	14.5	—	15.0	—	14.6

* In time sequence from left to right.

current flow is sufficiently accurate for the present experiments (where R can be determined to about $\pm 5\%$).

IV. THEORETICAL

The theoretical treatment of such results meets with various mathematical difficulties (Goldman 1943). There are further difficulties connected with the choosing of simple models (Johnson, Eyring, and Polissar 1954). Calculations of membrane potentials and resistances are possible only for very simple models, and then only with the aid of various unjustified assumptions. Divergences between the experimental data and the theoretical predictions are therefore difficult to deal with.

In the discussion which follows, models are considered in which a membrane separates two phases. Electric charge is assumed to be carried across the membrane only by the passive diffusion of ions. Such models may represent living cells if the active transport of ions in these cells does not transfer net charge across the membrane.

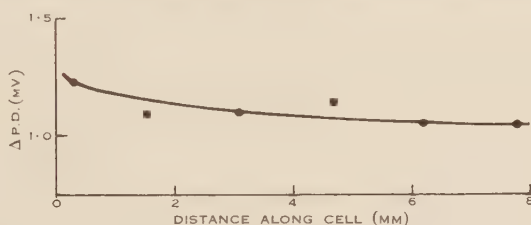


Fig. 7.—Change in the potential difference between a probe near the cell surface and the reference electrode, upon passing current across the cell surface, plotted as a function of the distance of the probe from the inserted micro-electrode.

(a) Membrane Potentials

In a system of two phases, containing only univalent ions, and separated by a membrane permeable to cations only, one can derive without arbitrary assumptions (Hodgkin and Katz 1949):

$$E_{oi} = (RT/F) \ln(\sum_j^+ P_j \cdot {}_o a_j) / (\sum_j^+ P_j \cdot {}_i a_j), \quad (3)$$

$$= (RT/F) \ln(C_o^+ / C_i^+), \quad (3a)$$

where

${}_o, {}_i a_j$ = activity in the o and i phase of the j th ion,

P_j = permeability of the membrane to the j th ion,

and

$$C_{o,i}^+ = \sum_j^+ P_j \cdot {}_o, {}_i a_j.$$

Superscript $+$ or $-$ signs indicate that the summation is taken over cations or anions only.

Equation (3a) is the general form of equation (1) already used. The permeability coefficient P_j contains the product of the mobility of the ion in the membrane and its partition coefficient between membrane and solution.

In calculations based on more difficult models, e.g. those in which the membrane is permeable to ions of both signs or to bivalent cations, it is usually assumed that

the partition coefficients are all unity. (A model for which this may be justified is one in which ions penetrate the membrane in regions of high dielectric constant.) Further simplifying assumptions are also necessary. There are three common assumptions:

- (i) Henderson's—that all ions have linear concentration gradients in the membrane.
- (ii) Goldman's—that the electric field has a linear gradient in the membrane.
- (iii) Planck's—that there is everywhere microscopic electroneutrality.

These lead to the following (different) equations:

- (i) Henderson's equation

$$E_{oi} = (RT/F) \left[\frac{\sum_j P_j z_j^{-1} (a_j - o a_j)}{\sum_j P_j (a_j - o a_j)} \right] \ln(C_o/C_i). \quad (4)$$

- (ii) Goldman's equation

$$E_{oi} = (RT/F) \ln[(C_o^+ + C_i^-)/(C_i^+ + C_o^-)]. \quad (5)$$

- (iii) Planck's equation

$$E_{oi} = (RT/F) \ln[(C_o^+ + p C_i^-)/(C_i^+ + p C_o^-)], \quad (6)$$

where

$$p = \frac{[\ln(A_i/A_o) - (FE_{oi}/RT)]}{[\ln(A_i/A_o) + (FE_{oi}/RT)]} \times \frac{[A_o - A_i \exp(FE_{oi}/RT)]}{[A_o \exp(FE_{oi}/RT) - A_i]},$$

and

$$A_{o,i} = \sum_j o_i a_j.$$

The nature of the expression for p makes the Planck equation insoluble except by trial and error. This is unfortunate, since it is based on a more plausible assumption than the others. The underlying assumption in the Henderson equation is physically impossible. Accordingly, we follow Johnson, Eyring, and Polissar (1954) in discarding the Henderson equation.

If the simple model (of a membrane separating two phases) is modified by the addition of a negatively charged Donnan phase separating phase o from the membrane, we have a model which may more closely represent the plant cell. Equation (5) is then used to find E_{wi} , the p.d. between the wall phase (w) and the i phase, and the following substitutions made:

$$E_{oi} = E_{ow} + E_{wi},$$

and

$${}_w a_j = {}_o a_j \exp(-z_j F E_{ow}/RT).$$

We then obtain:

$$E_{oi} = (RT/F) \ln \left[\frac{C_o^+ + C_i^- \exp(FE_{ow}/RT)}{C_i^+ + C_o^- \exp(FE_{ow}/RT)} \right], \quad (7)$$

where E_{ow} is the Donnan p.d. between the wall and the o phase. The effect of the anions on the overall p.d. is thus reduced by a factor of 10 for each 58 mV of Donnan potential of the wall.

If the anions are assumed to have vanishingly small permeabilities, equation (7) reduces again to equation (3). In this case estimates of a and $(K_i + a \cdot Na_i)$ from equation (1) are independent of the presence of a wall p.d., whether the Donnan system of the cell wall is in contact with the membrane or not.

(b) Membrane Resistances

Even in the simple case in which only univalent cations are considered, it is necessary to use simplifying assumptions to derive expressions for the membrane resistance. There is thus no simple rigorous equation corresponding to equation (3). The quantities measured in the present work are r and r_o , defined by:

$$r = (E'_{oi} - E_{oi})/J = V/J,$$

and

$$r_o = \lim_{J \rightarrow 0} (r),$$

where E'_{oi} is the p.d. when the current density is J , E_{oi} is the p.d. at zero current, and $V = E'_{oi} - E_{oi}$.

Expressions can be derived for r and r_o in terms of A_o , A_i , V , C_o , and either E_{oi} or C_i . Since E_{oi} is also measured in these experiments, its use is convenient. For the model involving permeability to univalent cations only, we find (using Goldman's equation):

$$r_o = \frac{RT[(1/C_o) \cdot (1/C_i)]}{F \ln(C_i/C_o)} \quad (8)$$

$$= \frac{R^2 T^2 [\exp(FE_{oi}/RT) - 1]}{F^3 E_{oi} C_o}, \quad (8a)$$

and

$$r = \frac{RTV[1 - \exp(FE'_{oi}/RT)]}{F^2 E'_{oi} C_o [1 - \exp(FV/RT)]}. \quad (9)$$

Using Planck's equation

$$r_o = \frac{RT \ln(A_i/A_o)[A_o - A_i \exp(FE_{oi}/RT)]}{F^2 C_o (A_i - A_o) [\ln(A_o/A_i) - (FE_{oi}/RT)]}, \quad (10)$$

and

$$r = \frac{V \ln(A_i/A_o)[A_o - A_i \exp(FE'_{oi}/RT)]}{FC_o (A_i - A_o) [FE'_{oi}/RT + \ln(A_i/A_o)] [\exp(FV/RT) - 1]}. \quad (11)$$

For the model involving univalent ions of both signs, the Goldman equations (8, 8a, and 9) are simply modified by writing $(C_o^+ + C_i^-)$ for C_o , and $(C_i^+ + C_o^-)$ for C_i .

If, as we assume in treating the experimental results, the important terms in C_o are $P_K \cdot K_o$ and $P_{Na} \cdot Na_o$, we can write

$$C_o = P_K(K_o + a \cdot Na_o).$$

It is then possible, using the Goldman equation, to calculate r for each cell from the parameters derived from the measurements of potential, i.e. E_{oi} and $(K_o + a \cdot Na_o)$, if a value is assumed for P_K . Fitting the experimental values for r then gives a value for P_K . If the Planck equation is to be used, a plausible guess must be made as to the value of A_i .

It is found that the following features distinguish the Goldman equations from the Planck equations:

- (i) In the Goldman equation, r_o varies more rapidly with K_o than it does in the Planck equation.
- (ii) The addition of a Donnan phase separating the o phase from the membrane reduces the range of r_o in the Goldman equation, but not in the Planck equation.
- (iii) The value of P_K calculated for observed values of r_o , α , and $(K_i + \alpha \cdot Na_i)$ using equation (8) is much greater than that using equation (10).
- (iv) In the Goldman equation, r varies more rapidly with J or E'_{oi} than it does in the Planck equation, i.e. the rectification effect is greater. However, r varies less rapidly with J or E'_{oi} in the presence of an added Donnan phase. In the Planck equation the rectification does not depend on the presence of a Donnan system.

V. DISCUSSION

(a) Potential Differences

(i) *Comparison with Theory*.—The results (cf. Figs. 2 and 3) have been compared with the predictions of equation (1), derived for a very simple model. It is assumed (1) that potassium and sodium have sufficiently large permeabilities or concentrations, compared with the other ions, that they alone determine the membrane potential difference; and (2) that the ratio of their permeabilities, and their activities in the cytoplasm, remain constant. When the two available parameters are selected for best fit, the agreement between results and calculation is generally good. This is not sufficient justification for assuming the correctness of the model, for equation (5) shows that under the conditions of these experiments a non-zero value of $P_{Cl} \cdot Cl_i$ cannot be distinguished from a non-zero value of $P_{Na} \cdot Na_o$. However, the results of experiments in which choline is substituted for sodium (Table 2) indicate that $P_{Cl} \cdot Cl_i$ is negligibly small, and that the model involving only potassium and sodium may be used. In mixtures of potassium and choline chlorides, the difference in p.d. between $K_o = 1.0$ and $K_o = 0.1$ mN is close to the value of 58 mV predicted for a potassium electrode (line (iv) in Table 2). Whether choline or sodium is present at a concentration of 0.1 mN affects the p.d. little (line (iii) in Table 2) since the product $P_K \cdot K_o$ is so much greater than the corresponding ones for choline or sodium. The different behaviour in sodium and potassium mixtures must then be due to the penetration of sodium, and not to the efflux of internal chloride ions.

The values of the parameters obtained using equation (1) are entirely plausible: $P_{Na}/P_K = \alpha = 0.06 \pm 0.01$ (mean of seven experiments \pm S.E.M.) (MacRobbie and Dainty calculate 0.05 for *Nitellopsis obtusa* from measurements of ionic fluxes into the "protoplasmic non-free space"), and $K_i + \alpha \cdot Na_i = 112 \pm 14$ mN (mean of seven experiments \pm S.E.M.) (typical values of the vacuolar concentrations of these ions are 80 mN and 50 mN for K and Na respectively (Hope and Waker 1960)). The concentrations of these cations in the vacuole are supposed by MacRobbie and

Dainty to equal those in the cytoplasm, but this rests on some assumptions. The value under discussion is thus not inconsistent with past findings.

This suggests that the model represents a good approximation to the true state of affairs. Accordingly we conclude that Osterhout was correct in attributing the resting potential to a diffusion potential for which potassium is chiefly responsible.

(ii) *The Cell Wall in Relation to the Membrane.*—The approach of the potential to equilibrium (Fig. 1(b)) has in most experiments a half-time of 3–5 min. From this time course one can calculate the time course of the potassium and sodium concentrations just outside the membrane. These are presumably the concentrations in the innermost layer of the cell wall. The half-times for the approach of these concentrations to the values for the bathing medium are found to be also in the region of 3–5 min. Crank (1956) has given graphical solutions for the problem of diffusion into a plane sheet. From his graphs it appears that the half-time for equilibration of the inner face of such a sheet is about twice the half-time for the equilibration of the *average* concentration in the sheet. Thus our measured half-times of 3–5 min for the concentration in the inner region of the cell wall correspond to half-times of $1\frac{1}{2}$ – $2\frac{1}{2}$ min for the average concentration of the whole wall. This is very similar to the value of 116 ± 13 sec found by Dainty and Hope (1959) for isotopic sodium exchange in isolated walls of this species, under comparable conditions. We conclude that sodium–potassium exchange in the neighbourhood of the plasma-lemma proceeds via the Donnan phase of the cell wall.

Measurements of the overall p.d. (E_{oi}) give no information as to the wall Donnan potential. Gaffey and Mullins (1958) erroneously concluded that the wall Donnan potential was low because they did not detect it during measurements of E_{oi} . The question of a possible intimate contact between the membrane and the Donnan phase of the wall will be discussed below.

(iii) *Deviations from the Model.*—Some results (Fig. 4), particularly those from cells which have been given prolonged pretreatment in sodium chloride, show consistent small differences from the calculations which are difficult to treat. In particular, the p.d. for $K_o = 0.1$ ($Na_o = 1.0$) is sometimes more positive than is predicted by a constant α and $(K_i + \alpha \cdot Na_i)$ derived from the other p.d. values (Fig. 4(b)). Another occasional deviation is a more positive p.d. in $K_o = 1.0$ ($Na_o = 0.1$), when the change in p.d. upon changing K_o from 0.7 (or 0.5) to 1.0 may exceed the “thermodynamic” value for a perfect potassium electrode (Fig. 4(a)).

In principle, the model might plausibly be modified in a number of ways:

- (1) K_i or Na_i might vary with K_o or Na_o ;
- (2) α might vary with E_{oi} , K_o , or Na_o ;
- (3) P_{Ca} might not be negligibly small; or
- (4) P_{Cl} might not be negligibly small.

These possibilities will now be discussed:

- (1) Some change in K_i or Na_i might occur during equilibration in solutions of varying K_o . This would give a divergence opposite to that observed in high K_o solutions, but might explain the divergence in low K_o . However, we favour the explanation next discussed.

- (2) A small increase in α (i.e. P_{Na}/P_K) would explain the divergence in low K_o solutions; since K_i is likely to be much greater than $\alpha \cdot Na_i$, the p.d. in high K_o will be very insensitive to α . As will be discussed later, an increase of α in low K_o is also suggested by the resistance measurements.
- (3) Under the conditions of the present experiments $P_{Ca} \cdot Ca_o$ should be negligible. A moderate value of Ca_i will add a constant term to the quantity $K_i + \alpha \cdot Na_i$ without altering the form of the curve for E_{oi} against K_o .
- (4) As has already been discussed, a non-zero value of $P_{Cl} \cdot Cl_i$ has been ruled out by the choline chloride experiments. If P_{Cl} is not zero, there will be a term $(P_{Cl}/P_K) \cdot Cl_o$ to be added to $K_i + \alpha \cdot Na_i$, but it is likely to be negligible under resting conditions. It seems possible, however, that the more positive potential observed in high K_o may be due to an increase in P_{Cl} , so that the term $(P_{Cl}/P_K) \cdot Cl_i$ may no longer be negligible compared with $(K_o + \alpha \cdot Na_o)$. This increase in P_{Cl} as E_{oi} decreases is a reasonable assumption if the initiation of the action potential is indeed due to an increase of P_{Cl} (Gaffey and Mullins 1958).

(iv) *Effect of Pretreatment*.—Almost no change in resting potential was observed when K_o was varied in media also containing 0.5 mN calcium ions. A model which would give this effect is one in which a Donnan phase (negatively charged) is in contact with the membrane, and separates it from the external medium. However, as will be discussed, this model does not fit the data for membrane resistance as a function of current density. The more likely explanation of the action of the pretreatment in allowing the observed changes in p.d. is that exchange tracks with mostly monovalent cations as counterions are established between a region near the plasmalemma and the medium. Before the pretreatment the counterions would have been almost entirely calcium and the exchange of a given number of monovalent cations along these paths would have been more difficult.

(b) Resistances

(i) *Comparison with Theory*.—When the data for E_{oi} as a function of K_o are fitted by the simple equation (1) in which α and $(K_i + \alpha \cdot Na_i)$ are constant, we can evaluate these two parameters for each cell. It is then possible to calculate for each cell the relative membrane resistance in each experimental solution, using equation (8) or (10) for r_o . Absolute values of r_o or r involve P_K as an additional parameter, which can be chosen for each cell for best fit. It is found that the experimental values for r_o agree qualitatively with the values calculated from Goldman's equation (8), but that the quantitative agreement is not good. For example the range of r_o as K_o goes from 0.1 to 1.0 mN is frequently about 3 : 1 (Fig. 6), while equation (8) predicts about 4.5 : 1 for most cells. Similarly, Planck's equation (10) predicts* a range of 2.5 : 1; again the fit is not exact, although it is better than equation (8).

When calculations are made of r as a function of current density (or, more conveniently, as a function of the change in membrane potential, V) the Goldman

* Assuming a plausible value for A_i .

equation (9) is very often an excellent quantitative fit to the data (Fig. 8). The Planck equation (11) predicts a much smaller change in r with V than is observed (Fig. 8). A similar observation was made by Goldman (1943).

Calculations from the Goldman equation applied to the model containing a Donnan phase resemble those from the Planck equation, as would be expected (Johnson, Eyring, and Polissar 1954). Thus they tend to fit the data for r_o better than the simple model, but are a worse fit to the data for r .

One factor which could reduce the range of r_o with concentration is an increase in P_{Na} (i.e. α) in solutions with relatively high sodium concentration. Indications of such an effect were mentioned above, in discussing the p.d.'s. From the results of two experiments, calculations were made of the variation in α needed to be consistent

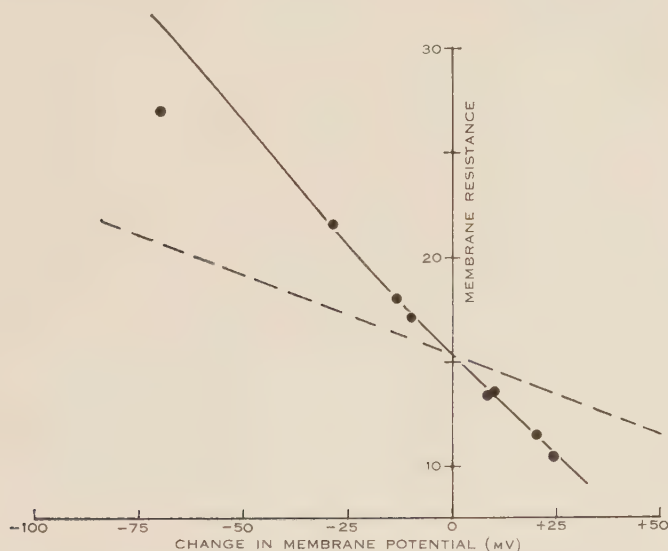


Fig. 8.—Membrane resistance r (in $k\Omega\text{ cm}^2$) as a function of the change in membrane potential produced by the applied current. ● Experimental points for one cell. — Fitted curve based on Goldman's equation (eqn. 8). - - - Curve based on Planck's equation (eqn. 11), fitted at $V = 0$ (at the resting potential).

with the observed range of r_o . To reduce this range from 4.5 : 1 to 3 : 1, α would have had to increase from 0.08 to 0.15 as Na_o approached 1.0 mN. In the second experiment the increase in α was from 0.05 to 0.14. The resting potential ($Na_o = 1.0$) would be very little different from that observed, as a result of such a change. Thus, the assumption of a constant α should be regarded as a first approximation only.

It is difficult to explain, on the present basis, the absence of a large increase in resistance on substituting choline for sodium. The increase was less than would be expected if α were as high as here suggested. The choline ion itself may penetrate, but the data for p.d. change on choline substitution (Table 2) suggest that the cell is much less permeable to choline than sodium.

In some experiments, not illustrated here, the resistance (r) observed during the passage of a depolarizing current was higher than that calculated. This may result from an increase in chloride permeability due to the depolarization. A similar explanation has been offered here for the extra depolarization sometimes observed in high potassium solutions.

(ii) *The Meaning of "Membrane Resistance".*—It is apparent from the results that qualitatively the membrane resistance is determined by the concentration of potassium, and, to a lesser extent, sodium. This largely explains the great variation in the published values for "membrane resistance" in the Characeae, since, amongst other things, the potassium concentration varied considerably amongst the published experiments. A comparison of this sort is given by Walker (1960). In these cells, too, there is little doubt that almost the whole of the observed resistance refers to a structure somewhere between the microelectrode in the flowing cytoplasm and the external medium, the contribution of the tonoplast being immeasurably small (see Walker (1960) for experiments bearing on this point made with *C. australis* as well as *Nitella* sp.). The most likely structure with the required properties is the plasmalemma.

(iii) *The Permeability of the Plasmalemma.*—By identifying the observed membrane resistance r_o with the theoretical resistance calculated from equations (8) or (10), and inserting values of a and $K_i + a \cdot Na_i$ from measurements of p.d., it is possible to calculate P_K and P_{Na} . Values from the present studies are $P_K = 10^{-5}$ and $P_{Na} = 10^{-6}$ cm sec $^{-1}$, if the Goldman equation is adhered to. This would suggest passive fluxes across the plasmalemma of the order of 10 p-equiv. cm $^{-2}$ sec $^{-1}$ for both K and Na, for an external medium containing 0.1 mN K and 1.0 mN Na. Using values of E_{oi} and fluxes of potassium and sodium reported by other workers using cells from the Characeae, the permeabilities to sodium and potassium can be calculated. Using the constant field assumption, the influx into phase i is (for values of E_{oi} greater than about 50 mV):

$$(\phi_j)_{o \rightarrow i} \simeq (-FE_{oi}/RT)P_j \cdot o a_j.$$

These values are compared in Table 4 with permeabilities from the present study. The latter are considerably higher. Clearly measurements of fluxes across the plasmalemma need to be made on the present cells, since the agreement of the permeabilities calculated from the two types of experiment would be useful evidence for the "constant field" model.

(iv) *Rectification by the Plasmalemma.*—It is interesting to see that rectification as observed is quite accurately predicted by the simple model of a membrane with unequal concentrations of ions on the two sides. Internal cations at high concentrations are able to carry positive current out of the cell with relative ease compared with external cations at lower concentration, carrying positive current into the cell when the current is reversed. The higher resistance when positive current is carried into the cell is successively reduced as the permeating ion (potassium) is increased relative to sodium (cf. Fig. 5).

Weidmann (1949) obtained somewhat different results with *Nitella*, but Findlay's (1959) data, also for *Nitella* sp., can be fitted qualitatively by the Goldman

relation, although he interprets his results as a sharp change in r as the applied current is reversed in direction.

(v) *Conductance by Anions*.—The experimental results reported here have been interpreted in terms of the permeabilities of the plasmalemma to potassium and sodium ions only. This has been reasonably successful, and it is concluded that the permeability to chloride, or to be precise its product with the chloride concentration, is generally negligible compared with the same quantities for potassium and sodium.

TABLE 4
COMPARISON OF PERMEABILITIES OF THE PLASMALEMMA IN VARIOUS CELLS

Species	Influx (p-equiv/cm ² . sec)		External Concentration (mN)		E_{oi} (mV)	Permeability (cm sec ⁻¹)		Reference
	K	Na	K	Na		K	Na	
<i>Nitellopsis obtusa</i>	4	8	0.65	30	-130	1×10^{-6}	5×10^{-8}	MacRobbie and Dainty (1958)*
<i>Nitella axillaris</i>	0.47	—	0.06	—	c. -150	1.3×10^{-6}	—	Diamond and Solomon (1959)*
<i>Chara globularis</i> (corticated)	2.8	0.7	1.4	10	-150	3×10^{-7}	1×10^{-8}	Gaffey and Mullins (1958)*
<i>Chara australis</i>	—	—	0.1	1.0	-155	10^{-5}	10^{-6}	Present work†

* Fluxes measured.

† Resistance measured.

(c) Conclusions

We have shown that, under suitable conditions, the p.d. and resistance of the plasmalemma of *C. australis* cells are largely determined by the passive diffusion of sodium and potassium ions across it. The present measurements of p.d. and resistance have given plausible values for the permeabilities of the membrane to sodium and potassium, and for the internal concentration parameter $K_i + a \cdot Na_i$. The results agree with the predictions of the Goldman equations, which involve the assumption of a linear potential gradient in the membrane and the neglect of phase boundary potential differences at the membrane surfaces. There are (contrary to the opinion of Johnson, Eyring, and Polissar 1954) models for which the neglect of the phase boundary p.d.'s may be justified. However, the linear potential gradient remains an arbitrary assumption, although widely accepted. It is possible that other models, based on a less arbitrary assumption, may yield similar equations, and this is being investigated.

For reasons discussed we suggest that the plasmalemma is in contact with a layer of solution with approximately the same ionic concentrations as the external medium, but is cut off from the latter by the Donnan phase of the cell wall, which governs the rate of approach to equilibrium on changing the external environment.

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PERONOSPORA TABACINA IN TOBACCO: TRANSPIRATION, GROWTH, AND RELATED ENERGY CONSIDERATIONS

By I. A. M. CRUICKSHANK* and N. E. RIDER*

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Summary

An investigation of the influence of *Peronospora tabacina* Adam on the transpiration and growth rates of the leaves of *Nicotiana tabacum* L. in three environments is described.

In the presporulation stage of infection the following results were obtained:

- (1) The main contribution to the enhanced total daily water loss from diseased plants occurred at night when the ratio of diseased to healthy plant transpiration approached 2.0.
- (2) Leaf growth was almost zero in the diseased plant from the third day after inoculation while growth in healthy plants continued at approximately 10% per day.

In the postsporulation stage of infection, transpiration from the diseased tissue decreased to less than that from healthy tissue, the exact ratio being controlled by the environment.

Temperature and temperature gradient measurements over healthy and diseased leaves at night showed that:

- (1) Temperature of presporulation diseased tissue was above that of healthy tissue by 0.1–0.2°C.
- (2) The transport of sensible heat from the air to the leaf surface was less for diseased than healthy tissue by an almost constant amount.
- (3) Energy balance considerations adequately accounted for the measured night transpiration from healthy material but only half the requirement for the diseased tissue.

The implication of the observed transpiration rates and of energy balance considerations are discussed.

I. INTRODUCTION

Blodgett (1901) observed that plants of *Rubus* sp. infected by *Gymnoconia interstitialis* (Schl.) Logh. transpired nearly twice as much as disease-free plants. Reed and Cooley (1912) working with *Gymnosporangium juniperi-virginianae* Schm. claimed that infection lowered the transpiration rate from apple leaves to one-quarter that of comparable healthy leaves.

A more complete analysis of the effect of rust infection on foliage transpiration was reported in a series of papers by Johnston and Miller (1934, 1940). They found that the overall transpiration of a susceptible wheat plant heavily inoculated with *Puccinia triticina* Eriks. at anthesis was increased by 17%, and that the diurnal rhythm of transpiration was seriously disturbed.

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Other studies concerning the influence of disease on transpiration include the effect of smut (*Ustilago tritici* Pers. Rostr.) (Kourssanov 1928) and the powdery mildews *Erysiphe graminis* f. sp. *hordei* (Graf-Marin 1934) and *E. polygoni* D.C. (Yarwood 1936) on their appropriate hosts, are in general agreement with the results of Johnston and Miller (1940).

The studies on leaf-infecting fungi referred to above have been confined to a demonstration of changes in transpiration rates in tissues in which sporulating lesions occurred. We report here an analysis of the effect of blue mould (*Peronospora tabacina* Adam) on the transpiration and growth rates of the tobacco leaf during the incubation phase of infection and following induction of sporulation. Preliminary measurements of air temperature gradients above an infected *P. tabacina* leaf surface are discussed in relation to the phenomenon of increased nocturnal transpiration of diseased leaf tissue.

II. MATERIALS AND METHODS

Three parallel series of experiments were conducted to measure the effect of *P. tabacina* on the transpiration and growth rate of the tobacco leaf under three different controlled environmental regimes. The glass-house conditions during the course of the experiments described below were:

Regime I: day temperature 20°C, day R. H. c. 48%; night temperature 15°C, night R. H. 50–70%.

Regime II: day temperature 25°C, day R. H. c. 35%; night temperature 18°C, night R. H. 35–50%.

Regime III: day temperature 31°C, day R. H. 40–42%; night temperature 21°C, night R. H. 42–50%.

The change from night to day temperatures and vice versa occurred at 0830 hr and 1630 hr respectively. Experiments were made under natural light conditions in July in Canberra (lat. 35° 20').

Tobacco plants (*Nicotiana tabacum* L. cv. Virginia Gold) of similar age and size (mean stem length 15 cm) grown under uniform conditions in 6-in. clay pots were used as the host material. The apical buds of the plants were removed and the leaf number reduced to three medium grown leaves. Five plant replicates and two pot blanks were used in each experiment.

Initially, the soil water in the pots was adjusted by watering to saturation and then allowing them to stand until excess water had drained away (Bliss, Kramer, and Wolf 1957). This level of soil moisture was maintained by daily addition of water, equivalent to the measured daily water loss. Water loss from surfaces other than that of the plants was prevented by enclosing each pot in a double polythene bag which was firmly tied around the base of the plant stem. Inoculation with a dense spore suspension of *P. tabacina* was done on a rotary turntable to ensure uniform infection of all leaves (Cruickshank 1958) and transpiration was measured gravimetrically.

Individual leaf areas were calculated as 60% of length by breadth measurements on the 3rd, 5th, and 7th days after inoculation. Leaf area as used in the

calculation of leaf growth rate is the sum of the individual areas of three leaves on each plant. Stem area was considered negligible in relation to leaf area and was ignored.

Transpiration is expressed as grams per square centimetre of leaf area (one surface only). Weighings were made at 0830 and 1630 hr from the 3rd-8th day after inoculation. On the 8th night uniform sporulation over the inoculated leaves was induced by returning the plants to a humidity cabinet (R.H. 97%, temp. 20°C)

TABLE 1
CHANGE IN TRANSPIRATION RATIOS DURING THE INCUBATION PHASE OF INFECTION
(PRESPORULATION)
Ratios of healthy to diseased transpiration given

Day	Time after Inoculation (hr)	Transpiration Period	Regime		
			I	II	III
4	72-88	Night	0.875	0.619***	0.718***
	88-96	Day	0.900	0.842	0.863*
5	96-112	Night	0.655**	0.529***	0.558***
	112-120	Day	0.912	0.984	0.914
6	120-136	Night	0.742*	0.514***	0.509***
	136-144	Day	1.025	1.202	1.026
7	144-160	Night	0.630**	0.530**	0.479***
	160-168	Day	1.134	1.212	0.943
8	168-184	Night	0.475***	0.457**	0.376***
	184-192	Day	1.562*	1.244	1.018
Significance of trend in transpiration ratios		Night	*		***
		Day	**	**	*

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

from 1630 hr to 0830 hr. Transpiration from the sporulating leaf tissue was determined over a further 24 hr after the plants had been returned to their respective environments and conditioned to them for 12 hr. For statistical analysis leaf area and transpiration data were subjected to logarithmic transformation. Results are presented in Tables 1 and 2 and Figures 1, 2, and 3.

Stomatal number per unit area of leaf was determined after fixing and clearing the leaf tissue with lactophenol (Berry 1959).

Temperature gradients above healthy and infected leaves exposed side by side at night in environmental regime III were measured by fine wire thermocouples. Figure 4 shows the apparatus clamped on the healthy leaf. Each leaf was held

between two "Perspex" frames. The top frame carried an inner smaller frame which supported the thermocouple wires of 48 S.W.G. (c. 0.004 cm dia.) copper and copper-nickel. The couples in each frame were connected differentially and the absolute temperature of one couple in each frame was measured with respect to a standard junction maintained in melting ice. The vertical separation between adjacent couples was 0.1 cm and the lowest was 0.1 cm from the bottom of the inner frame which was adjustable in height within the main frame. The couples were not placed one above the other in the vertical plane but were arranged in ascending order over a horizontal distance of 2.5 cm. This was an aid to construction and prevented the screening of any one by its neighbours. Leads were connected to one side of each upper main frame and terminated in a switchboard. An eye-reading reflecting

TABLE 2
MEAN TRANSPIRATION RATES OF DISEASED AND HEALTHY
TOBACCO LEAF IN IMMEDIATE POSTSPORULATION PERIOD
Transpiration rates given as $\text{g cm}^{-2} \text{ 24 hr}^{-1}$

Regime	Diseased Tissue	Healthy Tissue
I	0.105	0.111
II	0.084***	0.144***
III	0.123**	0.179**

** $P < 0.01$.

*** $P < 0.001$.

galvanometer was used to indicate the temperatures and temperature differences at and between the various positions in turn. Alternate runs for the two leaves were made about every 30 min over a period of several hours. Temperature differences could be read to 0.01°C and the absolute temperature to 0.1°C as the circuit resistances and galvanometer sensitivity were adjusted to give these resolutions.

III. RESULTS

(a) *Effect of Infection on Transpiration*

It was clear from inspection of the data that the 24-hourly transpiration from non-sporulating diseased tissue under each of the three environmental regimes was greater than that from healthy tissue growing under the same conditions. The transpiration response curves over 5 days of the incubation phase (72–182 hr after inoculation) of diseased and healthy tobacco leaves separated into night (1630–0830 hr) and day transpiration (0830–1630 hr) are illustrated in Figures 1 and 2. The ratio of the transpiration rate of healthy to diseased leaves over the same period of the incubation phase and the significance of trend in these ratios are presented in Table 1. No significant water loss occurred from the pot blanks.

The absolute magnitudes of the day transpiration were greater in all cases than those for the nights and for both day and night the transpiration levels increased with increasing temperature from regimes I to III. The transpiration rates were higher for the diseased than the healthy plants at night and the difference increased during the presporulation period. On the other hand, the transpiration

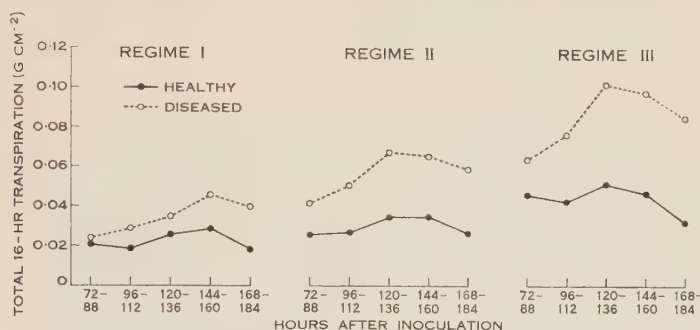


Fig. 1.—Night (1630–0830 hr) transpiration comparison between healthy and diseased plants (presporulation phase) in three environments.

rates during the day for the healthy and diseased plants were not very different but there was a definite trend towards a reduction in transpiration of the diseased relative to the healthy plants during the presporulation period. In Table 1 we give the ratios of healthy to diseased transpiration rates together with the statistical significances of the departure of the ratios from the expected value of unity.

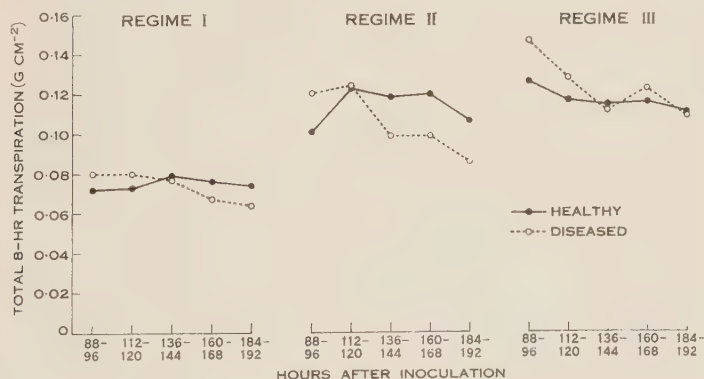


Fig. 2.—Day (0830–1630 hr) transpiration comparison between healthy and diseased plants (presporulation phase) in three environments.

Transpiration from diseased leaves over the 24-hr period before induction of sporulation was greater than that from healthy leaves. The mean total transpirations in the immediate postsporulation period are presented in Table 2. It is apparent from these results that transpiration of tobacco leaf tissue after *P. tabacina* had been induced to sporulate was not significantly different in regime I and was significantly less in regimes II and III than that of healthy tissue.

(b) *Effect of Infection on Leaf Growth*

To get a clear picture of the effect of *P. tabacina* on growth, young plants with rapidly expanding leaves were selected. Growth response curves of diseased and healthy leaves from the 3rd to the 7th day after inoculation under the three environmental regimes are illustrated in Figure 3. As for transpiration, earlier measurements were not possible as an initial 48-hr incubation period at high humidity and a 24-hr period for equilibration of the plants to their changed environment were required before measurements could be taken.

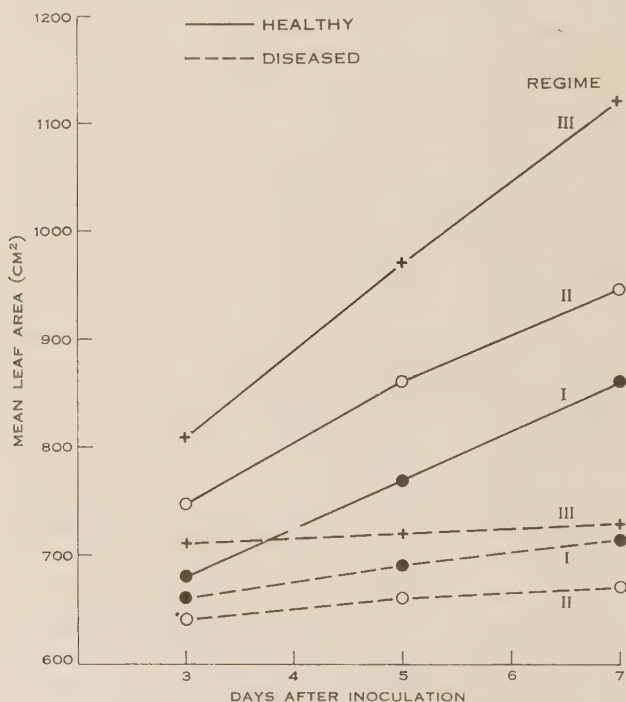


Fig. 3.—Leaf growth in healthy and diseased plants (pre-sporulation phase) in three environments.

In all instances the growth over the incubation period and over the same time interval for the healthy plants increased linearly with time. In terms of relative growth the diseased *v.* healthy slope comparisons within the same environments were significantly different ($P < 0.001$). Comparisons between healthy tissue subject to environmental regimes I and III showed a significant difference ($P < 0.05$) as was expected. For diseased plants the relative growth under environmental regime I differed significantly ($P < 0.01$) from those in the other two; regimes II and III were not significantly different in growths.

(c) *Possible Difference in Leaf Characteristics associated with Increased Transpiration: Stomatal Number per Unit Area of Leaf*

Mean counts of stomata per unit area (mm^{-2}) of leaf surface in both upper and lower epidermis (diseased 40.8, 177.0; healthy 39.3, 180.3 respectively) over

a series of 10 leaf samples show that in spite of the large difference in leaf areas, there was no significant difference between the diseased and healthy tissue (cf. Ziegenspeck 1944).

(d) Temperature Gradient Measurements above Non-sporulating Leaves

Figure 5 shows the result for two 20-min periods separated by about 2 hr. Each curve gives the mean for five separate measurements during the time intervals concerned. In both instances the temperatures at 0.8 cm from the leaf sur-

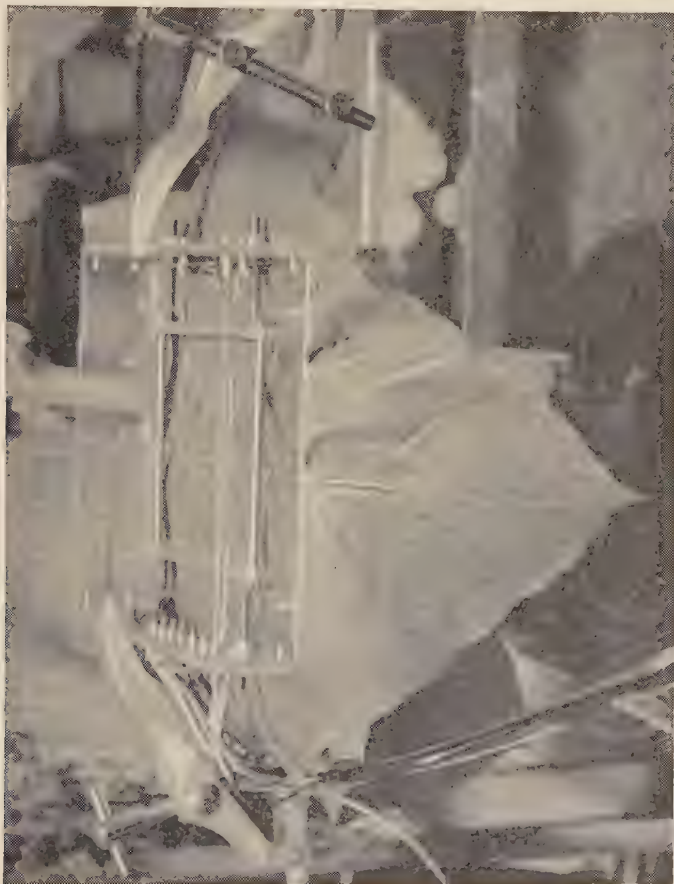


Fig. 4.—Method of clamping thermocouple apparatus against tobacco leaf.

faces were the same to within the accuracy of measurement (0.1 °C). In drawing the curves of Figure 5 it is assumed that these temperatures were identical although the subsequent argument would not be affected if they were not. In both these periods and in all others investigated but not illustrated the temperatures of the air immediately above the surfaces were higher over the diseased than over the healthy leaves. In the period 2030–2050 hr there was no definite temperature gradient above the diseased tissue but a marked gradient above the healthy tissue.

From 2220–2240 hr there were gradients above both, that above the healthy tissue being larger than that above the diseased. In all cases where the gradient existed there was a transfer of sensible heat from the air towards the leaf surface (i.e. down the temperature gradient).

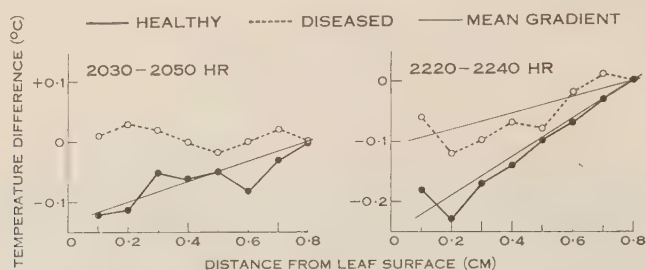


Fig. 5.—Examples of temperature profiles above healthy and diseased leaves in environmental regime III at 120–136 hr following inoculation.

(e) *Energy Considerations*

It is possible, by assuming that the heat transfer was due to molecular processes, to calculate the quantities being supplied from the air. The assumption that the processes are molecular rather than turbulent may not be valid but some support for this approach is given by the work of Vehrencamp (1953). He found that in the open at night in very calm and stable conditions close to a smooth natural surface half the actual heat transfer could be accounted for by assuming molecular

TABLE 3
SENSIBLE HEAT TRANSFER ($\text{CAL CM}^{-2} \text{ SEC}^{-1}$) TO LEAF SURFACE

Period	Diseased Tissue	Healthy Tissue	Difference
2030–2050 hr	0	1.05×10^{-5}	1.05×10^{-5}
2220–2240 hr	0.81×10^{-5}	1.92×10^{-5}	1.11×10^{-5}

transfer. In a closed glass-house in which the layers of air immediately above the leaf are stable and undisturbed it is not unrealistic to use the concept of molecular diffusion to indicate the likely magnitude of this transfer. Later, more evidence which suggests that molecular rather than turbulent processes were operative will be given. Taking the thermal conductivity of air as $6.14 \times 10^{-5} \text{ cal cm}^{-1} \text{ sec}^{-1} \text{ deg}^{-1}$ and drawing mean temperature gradients through the curves of Figure 5 the sensible heat transfer in the two periods has been calculated and is given in Table 3. We should note that these values represent the minimum transfer that could have taken place as the presence of turbulent processes would have enhanced the values. Further, gradients above one of the two leaf surfaces have been considered so to

arrive at likely figures for the total transfer the values given in Table 3 should be doubled. Thus it appears that the presence of disease in the tissue reduces the amount of sensible heat transfer from the air by about 2×10^{-5} cal cm⁻² sec⁻¹.

Although leaf surface temperatures were not measured the curves of Figure 5 suggest that the diseased leaf was *c.* 0.1–0.2°C higher than the healthy leaf. This implies that the outgoing radiation from one surface was about 1.5×10^{-5} cal cm⁻² sec⁻¹ greater for the diseased than the healthy leaf. Considering both surfaces the excess radiative heat leaving the diseased leaf was about 3×10^{-5} cal cm⁻² sec⁻¹.

As the general environment of both leaves was the same an extra energy supply of about 5×10^{-5} cal cm⁻² sec⁻¹ must have been available to the diseased leaf on these accounts. To this must be added the energy required to maintain the enhanced transpiration rate. The temperature observations reported were made in the period 120–136 hr after inoculation when, according to Figure 1, the diseased and healthy leaves transpired *c.* 0.1 and 0.05 g water per cm² leaf surface respectively in the 16-hr night. This represents an average energy consumption of *c.* 10×10^{-4} and 5×10^{-4} cal cm⁻² sec⁻¹ respectively. The consideration above shows that only a very small fraction of these energy requirements was satisfied by extraction of heat from the air.

However, since the glass-house was heated the temperature of the inside of the roof was near to the general ambient temperature. Assuming this surface to be at 20°C and the leaf surface temperature to be at 16.5°C we may calculate the net gain of radiative energy to the upper surfaces of the leaves. The temperature observations indicate that the leaf surfaces were in the region of 16.5°C but there was some fluctuation throughout the predominantly clear night. Considering the surfaces in question to radiate as black bodies the net gain to the leaves amounted to about 4.5×10^{-4} cal cm⁻² sec⁻¹. There would have been little or no net transfer of radiant energy to the undersurfaces of the leaves. Thus the energy requirement necessary to sustain the observed transpiration rate from the healthy leaves was provided in the main by radiative exchange. There was a small contribution of heat directly from the air and presumably also from the respiratory process.

For the diseased leaf we can offer no such satisfactory explanation. The energy required for about half the observed rate of transpiration is provided by radiative exchange but it is difficult to envisage any exchange process external to the tissue itself which could supply the deficiency. One is therefore forced to consider the energy released in the respiratory process as a possible source.* The dry weight of the plants was in the region of 5 g and each milligram of diseased tissue would be required to liberate 70 mm³ CO₂/hr/mg dry weight in order that the extra energy required could be released. In arriving at this value it is assumed that the reaction



was operative, that the plant tissue consisted of hexose alone, and no energy was consumed in growth and synthesis. A respiration and corresponding release of

* It has been suggested to us that if the water transpired originated from the soil, then there would have been a transport of heat to the leaves. Calculation shows that even if this water arrived in the leaf at its day temperature (31°C) then, in falling to 16°C, the quantity of heat made available for the diseased leaf would be *c.* 3.0×10^{-5} cal cm⁻² sec⁻¹ or about 3% of the total requirement.

energy at this rate was quite impossible as a weight of hexose in excess of the dry weight of the plant would have been consumed in the one night. However, the rate of growth of the healthy plant was about 10% and that of the diseased plant less than 1% per day at this time. A 10% increase in leaf area corresponded to a dry weight increase of 0.5 g per day. The energy liberated by 0.5 g hexose in oxidation in accordance with the above reaction is about 1.9 kcal. This energy spread throughout the night over the plant leaf area represents 3.3×10^{-5} cal cm² sec⁻¹, or less than 10% of the extra requirement.

Support for the assumed molecular nature of the heat transport is obtained from a consideration of the water vapour transfer away from the leaf surface. Making the assumption that the air in immediate contact with the leaf surface is saturated (it may contain somewhat less water than this) and that at a distance of 2 cm from the surface the water vapour content has fallen to the ambient level (about 45% R.H.), the amount of vapour transferred may be calculated. Taking the diffusivity for water vapour as 0.25 cm² sec⁻¹, the amount of water leaving each surface of the leaf (top or bottom) is 1.03×10^{-6} g cm² sec⁻¹ and the energy used in evaporating this was 6.2×10^{-4} cal cm⁻² sec⁻¹. For the two surfaces this amounts to 1.2×10^{-3} cal cm⁻² sec⁻¹. This very approximately is the energy used in the diseased leaf as calculated from the 16-hr water loss.

IV. DISCUSSION

The downy mildews caused by leaf-infecting members of the Peronosporaceae constitute a large group of economically important diseases which as a whole are very sensitive to the water relations of their hosts and their environments (Yarwood 1956). Sporulation of *P. tabacina*, the causal organism of downy mildew of tobacco (blue mould) is closely dependent on the diffusion pressure deficit of infected leaf tissues and the relative humidity of the ambient air in the immediate vicinity of the leaf surface (Cruickshank 1958).

Although the overall daily transpiration of infected tobacco leaf is greater than that of healthy leaf tissues it seems clear from the foregoing data that the increase in the transpiration of infected leaves prior to sporulation is very largely due to the higher rate of transpiration of infected plants during the night. The higher overall rate of transpiration of diseased plants could be expected to disturb the water economy of the host plant which under some field situations could influence the growth and quality of the tobacco leaf. Under near optimal relative humidity conditions for the sporulation of *P. tabacina* (Cruickshank 1958), the higher transpiration rate of diseased tissues during the night could produce conditions in the leaf sublamina layer favourable for sporulation. The former situation would be directly unfavourable for the growth of the tobacco plant. The latter condition would influence the reproduction of *P. tabacina* and thus play an important role in the epidemiology of blue mould of tobacco.

Previous workers studying foliage infections have confined their experiments to tissues exhibiting normal disease symptoms, viz. sporulating lesions. Studies of *P. tabacina*-infected leaf, during the 24 hr after sporulation had been induced, showed

that transpiration was either equal to or less than that of healthy plants grown under similar conditions (Table 2). This result is in agreement with that of Reed and Cooley (1912) but is in direct opposition to most of the literature in this field. It is suggested that this difference is due to the physically different nature of tobacco leaf tissue in comparison with that of cereals studied by most other workers, and to its rapid physiological collapse after sporulation. This is supported at least in part by Schramm and Wolf (1954) who, in a study of the transpiration of tobacco infected with black shank (*Phytophthora parasitica* var. *nicotiana* (Breda de Haan-Tucker)), state that water loss decreases as the disease progresses.

Reduction in plant growth due to disease has been reported by several authors (Graf-Marin 1934; Johnston and Miller 1934). The most outstanding feature of the growth data presented in Figure 3 is the almost complete inhibition of leaf expansion and the rapidity with which it occurred after infection. This effect on growth probably explains the distortion of young partially infected leaves which are sometimes observed in the field. It could also be responsible for the characteristic downward curving of unilaterally infected leaf petioles, and for the stunting of tobacco plants which have become systemically infected at an early stage of growth.

Several authors have speculated on the reasons for the higher rate of transpiration from diseased tissues. A change in the distribution and number of stomata was claimed by Dodge (1923). Graf-Marin (1934) attributed the increase mainly to an increase in the opening of stomata in diseased leaves. Johnston and Miller (1940) considered the higher rate of transpiration of diseased plants at night was due partially to transpiration through ruptures in the cuticle caused by sporulating uredia, in the case of the leaf rust (*P. tritici*) which they studied, and partially to the transpiration of the fungus itself. Stomatal number, cuticle rupture, and direct transpiration from external fungal conidiophores or other reproductive structures were not demonstrated in the present studies to be important contributing factors to greater transpiration. It must be concluded that in the present instance nocturnal transpiration prior to sporulation is primarily cuticular.

In a recent comprehensive review Raschke (1960) considers the factors which influence the energy balance and transpiration rates of individual healthy leaves. However, we believe the present paper to be the first attempt to investigate the different energy requirements of healthy and diseased leaves in identical environments. In the previous section we showed that the energy requirements necessary to sustain the observed transpiration rates from healthy tissue were satisfied. We were unable to suggest how the extra energy required for the enhanced transpiration from diseased tissue was obtained from external sources. We can only conclude that some other energy source is available associated with the presence of the fungus in the leaf tissue. According to Allen (1954) diseased tissue shows an increased respiration which he attributes to the utilization of energy-rich phosphate which permits a more rapid aerobic breakdown of carbohydrate. We see here that this explanation is not tenable as complete breakdown of all available carbohydrate does not give the required energy level necessary to maintain the enhanced transpiration rate. We are not able at the present stage to account, on an energy basis, for the high transpiration observed.

One characteristic of the curves of Figure 5 for the period 2220–2240 hr requires comment. It will be noted that the minimum temperature measured occurred not at the 1-mm level but at the 2-mm level and that the temperature distribution is reminiscent of that found by Lake (1956) above bare soil on very calm nights in the open. Normally it is considered that the only important source of radiative heat loss is the surface itself, in our case the leaf surface. However, it has been suggested (Ramanathan and Ramdas 1935; Rider and Robinson 1951) that under very stable conditions the air layer in contact with the surface may be in radiative rather than conductive equilibrium with its surroundings, the eddy conductivity approaching the molecular value. The unusual density distribution is remarkable and its maintenance for long periods not easily explained. However, for our present purpose it is sufficient to note that this type of temperature distribution in very stable conditions is not unknown and its existence may indicate the onset of molecular diffusion as we have assumed in our calculation above.

These simple and preliminary measurements of microenvironment reported here at least serve to show that the presence of disease in the leaf exercises some control. We hope to undertake a further more comprehensive investigation along these lines in the hope of providing a satisfactory energy balance at least for individual leaves.

V. ACKNOWLEDGMENTS

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GERMINATION OF *PERONOSPORA TABACINA*: EFFECT OF TEMPERATURE

By I. A. M. CRUICKSHANK*

[Manuscript received July 4, 1960]

Summary

In an investigation into the effect of temperature on the germination and germ-tube length of *Peronospora tabacina* Adam using an *in vitro* technique the following results were obtained:

- (i) Some germination occurred after incubation for 1 hr over the temperature range 20–30°C, while after 12 hr there was no significant difference in germination response over the range 8–27°C.
- (ii) Germ-tube growth was initially most rapid at the temperatures 24–27°C; however, after 12 hr, greatest growth had occurred over the temperature range 15–17.8°C.
- (iii) Multispore isolates of *P. tabacina* from Canberra and Parada, N. Qld., could be distinguished from an isolate from Manjimup, W.A., on the basis of differences in their percentage germination and germ-tube length after incubation for 5 hr.

The significance of temperature as a factor effecting germination and its relationship to the epidemiology of blue mould (*P. tabacina*) under field conditions is briefly discussed.

I. INTRODUCTION

Angell and Hill (1931, 1932) reported that the germination of freshly detached conidia of *Peronospora tabacina* Adam in water on glass slides incubated at 16–18°C for 24 hr was very uneven. This variability in germination in water of *P. tabacina* has been confirmed by several workers, especially Clayton and Gaines (1945). Wolf *et al.* (1934), Armstrong and Sumner (1935), and Clayton and Gaines (*loc. cit.*) make reference to the effect of temperature on the germination of North American isolates of *P. tabacina* but made no detailed studies in this field. No quantitative temperature studies in relationship to the germination of Australian isolates of this fungus have been reported.

In this communication is described a study of progressive germination and germ-tube growth of one isolate and the comparative germination and germ-tube growth of three Australian isolates over the temperature range 0–35°C.

II. MATERIALS AND METHODS

Preliminary experiments indicated that germination of *P. tabacina* was stimulated by drops of water which had been in contact with tobacco leaves for several hours (*cf.* Brown 1922). An aqueous solution of this unknown factor was obtained by placing drops of sterile water onto leaves of young tobacco plants in a humidity cabinet at 20°C. After 24 hr the drops were removed, bulked, and centrifuged to remove any solid matter. The supernatant was stored at –15°C.

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Conidia to be tested for germination were prepared by removing non-sporulating infected leaf areas from plants and incubating them overnight under optimal conditions for sporulation (Cruickshank 1958). Conidial suspensions were prepared by shaking the sporulating leaf tissues in sterile water and filtering them through sterile muslin. The suspensions were then centrifuged at 2000 r.p.m. for 2 min and the supernatants decanted. The conidia were resuspended in sterile water and the concentrations adjusted to *c.* 50,000 conidia per ml. Test tubes of melted "Bacto" prune agar (6 ml) were seeded with 1.5 ml of conidial suspension in a water-bath at 40°C. The seeded agar was held in the bath for 30 sec and then poured into 9-cm dia. petri dishes. Aliquots of 0.5 ml of the germination stimulant, and five blocks (5 by 5 by 1 mm) of the seeded agar were dispensed into watch-glasses (4-cm dia.). Each watch-glass was placed in an individual small petri dish. In order to prevent germination prior to the initiation of treatments all preparative operations were carried out at 4°C and the conidia suspensions and seeded agar were stored at 0°C when not actually in use.

Petri dish units containing watch-glass, seeded agar blocks, and ambient solution represented the basic test unit in the experiments described in the next section. Incubators ranging from 1.5 to $34 \pm 0.5^\circ\text{C}$ were used for the temperature treatments. At the cessation of the exposure periods the conidia were killed with 1% formalin and stained with 1% cotton blue in aqueous solution. The agar blocks were then examined microscopically. Percentage germination was determined on a quantal basis by recording the number of spores germinated in the first 100 spores counted per agar block. Germ-tube length was determined by classifying the first 10 germ tubes from each agar block on the basis of their lengths into five classes with the aid of an eyepiece graticule calibrated in microns (1 division = 108 μ).

The germination data was analysed using the angular transformation. The square-root transformation was used for the analysis of the germ-tube length data.

III. EXPERIMENTAL

(a) *Effect of Temperature and Time Combinations on Germination Response and Germ-tube Length*

Conidia of the Canberra isolate of *P. tabacina* were used in this experiment. Petri dish units were prepared as described above and seven were placed in incubators at each of the following temperatures: 1.9, 4.0, 8.6, 15.2, 17.8, 20.4, 23.9, 27.2, and 30.6°C. They were withdrawn individually from each incubator in the series after 1, 2, 4, 6, 8, 10, and 12 hr and the spores immediately killed. Results of this time course study are graphically illustrated in Figures 1 and 2. The three-dimensional diagrams represent the change in percentage germination and germ-tube length as temperature is increased over the range 1.9–30.6°C and the times of exposure to the temperatures within the range are increased from 1 to 12 hr.

Examination of the germination data showed that after 1 hr some germination had occurred at temperatures between 20.4 and 30.6°C. The percentage at 27.2°C was, however, significantly greater ($P < 0.001$) than that at any other temperature.

After 2 hr the percentage at 27.2°C was still significantly greater ($P < 0.01$) than that for any other treatment. At temperatures below 20°C and above 30°C there was at this time less than 50% germination while no germination had occurred at 1.9 or 4°C. The responses at the end of the 6-hr exposure could be grouped into five classes, viz.

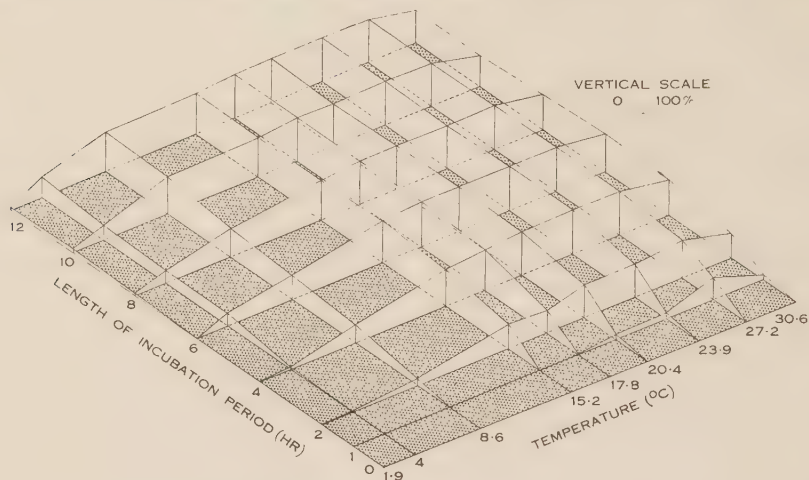


Fig. 1.—Relationship between mean germination response, temperature, and time.

1.7°C (zero germination); 4°C (28.4%); 8°C (50%); 15.2–27.2°C (88% mean value); 30.6°C (70.4% germination). The differences between classes were significant ($P < 0.001$). At the end of the experiment (12 hr) the range over which no significant difference ($P < 0.01$) in response occurred extended from 8–27°C.

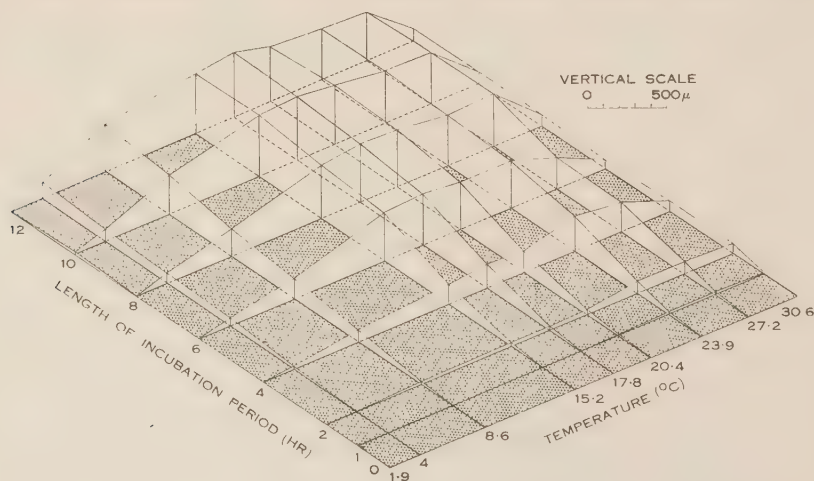


Fig. 2.—Relationship between mean germ-tube length, temperature, and time.

The analysis of germ-tube length data showed many similarities to the germination data but also some important differences as can be seen from a comparison of Figures 1 and 2. In the first hour greatest growth occurred at 23.9–27.2°C. Some growth, however, occurred over the whole 20.4–30.6°C temperature range.

After incubation for 2 hr germ-tube length had increased with temperature over the range 8.6–27.2°C. At the end of 4 hr, germ-tube length at 30.6°C had not significantly increased beyond growth at 2 hr while maximum growth had occurred at 23.9°C. The growth pattern after 6 hr showed that after this period of time germ-tube length at the lower temperatures, 15.2–20.4°C, had increased proportionally to that of germ-tubes at 23.9°C or higher temperatures, with the result that a broad optimal temperature range 15.2–23.9°C for germ-tube growth was well defined. With increasing incubation time the optimal temperature range for growth fell and became less broad. At the completion of the experiment (12 hr) the optimal temperature range for growth was 15.2–17.8°C. Satisfactory growth, however, occurred over the much wider range of 8.6–27.2°C.

(b) *Comparison of the Effect of Temperature on Percentage Germination and Tube Length on Three Isolates of P. tabacina*

Multiconidia isolates of *P. tabacina* from Canberra (originally from Ovens Valley, Vic.), Parada, N. Qld., and Manjimup, W.A., were grown over two generations under standardized environmental conditions in the glass-house on similar plants of *Nicotiana tabacum* L. cv. Virginia Gold. Cross-contamination was prevented by use of separate humidity cabinets at the time of inoculation and by the maintenance of humidities unfavourable for sporulation (Cruickshank 1958) over the incubation period in the glass-house.

From the results in Section III(a) it was apparent that given a long enough incubation period, a very wide range of temperature was satisfactory for germination. In order to sharpen the comparison between isolates an incubation period of 5 hr was chosen in this experiment. Results are presented in Figures 3 and 4 where the mean percentage germination and the mean germ-tube length are plotted against temperature respectively. Hand-fitted curves were drawn with due allowance given to the statistical analysis of the data.

Figures 3 and 4 show the similar behaviour of the conidial isolates from Canberra and Parada and the general similarity in the shape of the response curves of all three collections in terms of both germination and germ-tube length. In terms of the temperature levels used in this experiment the lower limits of the optimal temperature ranges were the same for all isolates. The upper limit of the ranges of the Manjimup collection, however, sharply distinguished this isolate from the other two. For germination, the optimal temperature range for conidial isolates from Canberra and Parada was 14.5–27.2°C while that from Manjimup was 14.5–23.9°C. For germ-tube length the optimal temperature range for the isolates from Canberra and Parada was 17.8–23.9°C and that for the Manjimup isolate 17.8–20.6°C. Differences in the levels of maximum germination and germ-tube length between the Manjimup and the other two collections were highly significant. The behaviour of the Manjimup isolate was confirmed in a completely independent subsequent experiment.

(c) *Reliability of Experimental Techniques*

(i) *Biological*.—In temperature studies of this type where germination must be prevented during the preparatory period by working at low temperature there

is necessarily a time lag between the initial temperature and the test temperature. In the present studies precautions were taken to reduce this time lag but it was

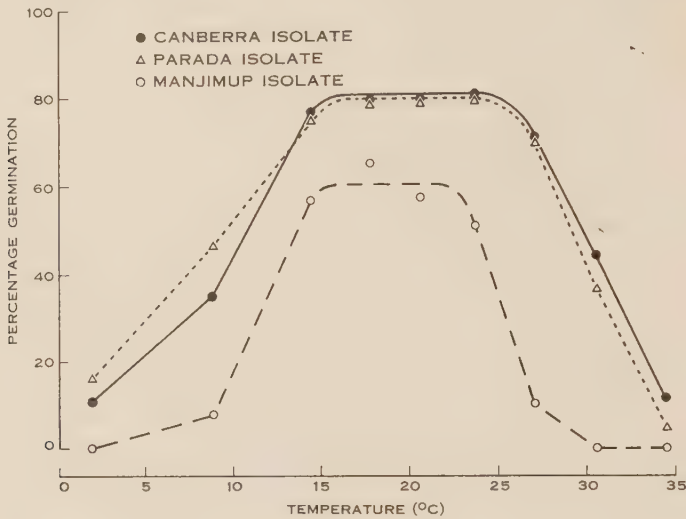


Fig. 3.—Germination response comparisons of three conidial isolates of *P. tabacina*.

not completely prevented. Measurements of the time necessary for the air within closed containers to come to equilibrium with test temperatures showed that the

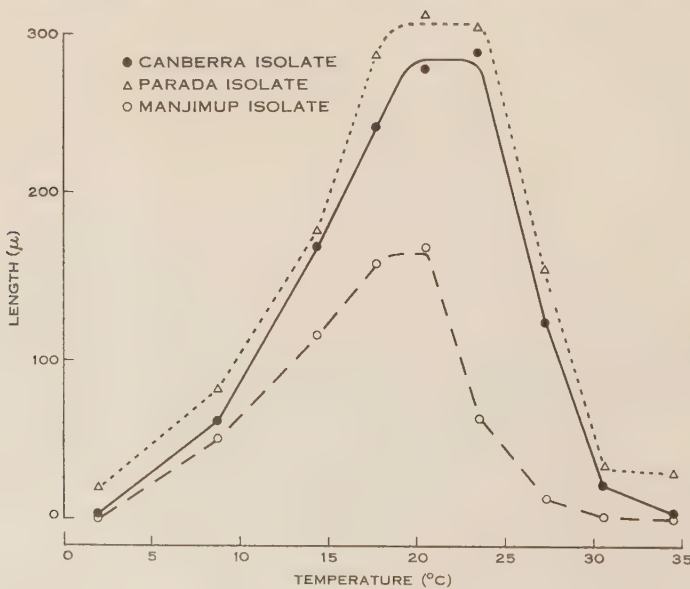


Fig. 4.—Germ-tube length comparisons of three conidial isolates of *P. tabacina*.

temperature adjustment followed a normal hysteresis curve. The time necessary for equilibrium was of the order of 10-15 min. The period at an intermediate temperature may have influenced the results in the shortest exposure (1 hr). It is

unlikely to have significantly affected the responses recorded after longer intervals of time.

(ii) *Statistical*.—For the germination data, the variability is most effectively expressed in terms of the binomial variation one would expect if all spores germinated separately instead of in groups in agar cubes. The data were analysed using the angular transformation, and the error variances for 100 spores per replicate were 17.17 and 12.82 in the two germination experiments in contrast to the expected value of 8.21. This indicates that the replicate variance for the 100-spore unit is 2.09 and 1.56 times greater than one would expect from chance alone and would imply the following replicate standard deviations for treatments:

Treatment Mean Germination	Replicate Standard Deviation	
	Expt. A	Expt. B
30%	6.63	5.72
50%	7.23	6.25
70%	6.63	5.72
90%	4.32	3.75

The variability in tube length increased with the mean. A square-root transformation was satisfactory in making replicate variation within treatments uniform. From the analysis one can estimate variances in the original units. Coefficients of variation between mean tube lengths of 10 tubes per replicate were:

Mean Tube Length (μ)	Coefficient of Variation
108	14.2%
216	11.9%
324	8.2%

IV. DISCUSSION

On account of the *in vitro* character of the germination test described above the germination response and germ-tube lengths measured cannot be considered identical with the germination behaviour *in vivo* of *P. tabacina*. It is assumed, however, in this discussion that the results in relationship to temperature closely approximate to the *in vivo* behaviour as all other environmental and nutritional factors were held constant. Variation in replicate behaviour in the present technique did not exceed that normally associated with biological material.

Wolf *et al.* (1934) demonstrated that some germination was accomplished at 7.2–15.5°C within 2 hr and that at 21°C 50–59% germination occurred after 5 hr. Conidia maintained for 22 hr at 26°C did not germinate. Armstrong and Sumner (1935) using incubation periods of 22 and 48 hr reported the optimum germination range of *P. tabacina* to be 15–23°C. No germination was reported at temperatures higher than 29°C. Clayton and Gaines (1945) conducted a series of germination tests over undefined incubation periods on several isolates of *P. tabacina*. They stated that the germination response of their isolates fell into two distinct classes which had optimal temperature ranges of either 1.5–10°C or 17.7–26.1°C.

The points of major importance that became apparent from the time course analyses were, firstly, the rapidity with which germination occurred under favour-

able conditions and, secondly, the role of the time factor in determining the shape of the consecutive temperature response curves (see Figs. 1 and 2). Initially germination occurred most rapidly at 27.2°C. However, after 6 hr, there was no difference in germination over the range 15.2–27.2°C while after 12 hr the range had increased still further from 8.6 to 27.2°C. Initially germ-tube growth occurred fastest at the higher temperatures of the range studied. The advantage gained was not, however, maintained over longer periods, thus indicating that these temperatures were not the true optima for growth. The optimal temperature range for growth after 12 hr was in fact 15.2–17.8°C—approximately 10 degrees lower than the temperatures which initially most rapidly stimulated it.

The optimal temperature range for germ-tube length over shorter periods is more narrowly defined than that for germination over the same periods. This is well illustrated in Figures 3 and 4. After 12 hr, however, although the optimal temperature range for germ-tube growth is very narrow, the range over which satisfactory growth occurs more nearly coincides with the optimal temperature range for germination.

Figures 3 and 4 show that the three Australian isolates of *P. tabacina*, representing isolates of this plant pathogen from three widely geographically separated areas, can be placed into two classes on the basis of germination and germ-tube length responses. In terms of the temperature levels used in these experiments the actual values for germination are 14.5–27.2°C and 14.5–23.9°C while those for germ-tube length are 17.9–23.9°C and 17.9–20.2°C. Although the ranges overlap, the very sharp fall in response at the end of the optimal ranges clearly indicates the biological reality of the two classes. Wark *et al.* (1960) have recently shown the presence of distinct ecotypes within the Australian *P. tabacina* population. Clayton and Gaines' results and those reported here could be explained on this basis.

The optimal temperature range or ranges for germination of *P. tabacina* are only of importance in terms of epidemiology when the time factor is also considered. Thus a longer period at some of the lower temperatures studied was equally as satisfactory as short periods at the higher temperatures. Conversely short periods at low temperatures were unsatisfactory. Germination under field conditions is complicated by the necessity of visible moisture on the leaves (Hill 1957). Temperature conditions necessary for germination in the field must coincide with adequate moisture conditions.

Our knowledge of the wide optimal temperature range for germination would suggest that the duration of optimal moisture conditions for germination may be a more important limiting factor than temperature. According to unpublished observations by the author in the Ovens Valley, Vic., during the 1959–60 tobacco-growing season, moisture conditions favouring germination were of the order of 5–6 hr in duration on cool clear nights. Temperature records over the same period showed that minimum night temperatures were frequently close to the lower limits of the optimal temperature range for germination after 6 hr, namely 10–15°C. Under these conditions temperature was unlikely to be a serious limiting factor either for germination or germ-tube growth.

In this discussion environmental conditions at night have been emphasized as this is the period of dew formation on leaf surfaces. Showery periods during daylight hours associated with cloudy conditions may, however, also provide moisture conditions favourable for germination. Under these conditions when temperatures are higher ($>20^{\circ}\text{C}$) germination occurs very rapidly as can be seen from Figure 1. Thus day temperatures as well as night temperatures may play a role in the germination of *P. tabacina* and consequently in the epidemiology of blue mould of tobacco.

Wark *et al.* (loc. cit.) have shown that isolates of *P. tabacina* vary in their sporulation capacity. The work reported here shows that germination and germ-tube growth are two further physiological characters which may be used to differentiate between strains of this fungus. The ecological advantage due to the differences demonstrated is not apparent in terms of long-term mean temperatures of the districts of origin of the isolates. There may, however, be factors not accounted for in normal meteorological records which have influenced the evolution of these differences.

V. ACKNOWLEDGMENTS

The author wishes to thank Messrs. G. A. McIntyre and M. L. Dudzinski, Division of Mathematical Statistics, C.S.I.R.O., for their examination and analysis of the data.

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A THIAMINE-REQUIRING MUTANT OF THE TOMATO

By J. LANGRIDGE* and R. D. BROCK*

[Manuscript received July 29, 1960]

Summary

A spontaneous, single-gene mutant of the tomato is shown to be unable to synthesize thiamine. The specific reaction lost is either the methylation of position 2 or the activation of position 5 of the pyrimidine ring.

I. INTRODUCTION

Apart from a note (Langridge 1955) on certain growth mutants of *Arabidopsis*, there has been no description in flowering plants of mutants of the *Neurospora* type; i.e. mutants which can be shown to be lethal in the absence of a specific organic compound. This report briefly describes such a mutant in the cultivated tomato, *Lycopersicon esculentum* Mill.

II. MATERIAL AND METHODS

The mutant studied is a spontaneous one occurring in two tomato lines, 11-1-1 and 25-1 (Giles and Hutton 1958), which were being bred for disease resistance. These two lines, now known as Merbein Mid-Season and 25-B had as common parents the varieties South Australian Dwarf and Hawaiian Experiment Station Line No. 4242. The mutant has not been found in any of the parent varieties.

The segregation of mutant plants observed in the selfed progeny of 110 heterozygous plants was 1387 wild-type to 450 mutant plants. Thus the mutant phenotype results from a single gene change (χ^2 for 3 : 1 = 0.24, $P > 0.5$). The mutation is completely recessive to its wild-type allele and neither somatic nor germinal back-mutation has been observed.

Plants homozygous for the mutation may survive for several months under good growing conditions, but they grow extremely slowly. The cotyledons are normal in size, shape, and colour, presumably because of the diffusion of metabolites into the seed from the heterozygous maternal parent. The few leaves formed by the mutant are small and at first pale yellow in colour. Later the leaves may become light green, but they soon lose their chlorophyll and turn white except at the main veins which remain green. After the chlorophyll is lost, the leaves wither and die. Frequently the main shoot apex also dies and short secondary shoots develop. Plants after about four months' growth are only 3-6 in. in height, very chlorotic but still alive. There is some variation in phenotype depending on the time of sowing, but experiments showed that this variation was not correlated with temperature or with light intensity during growth.

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Mutant plants were tested for their response to organic substances by spraying them daily with nucleic acid hydrolysate, casein hydrolysate, water-soluble vitamins, and organic acids. The preparation, composition, and concentrations of the supplements are described by Langridge (1958). Three plants were sprayed with each supplement.

III. RESULTS AND DISCUSSION

The only effective treatment was the vitamin mixture, which produced a pronounced greening of the tissue after 2 days and a complete disappearance of the deficiency symptoms after 5 days of application. The active vitamin of the mixture was shown to be thiamine by means of the screening test described by

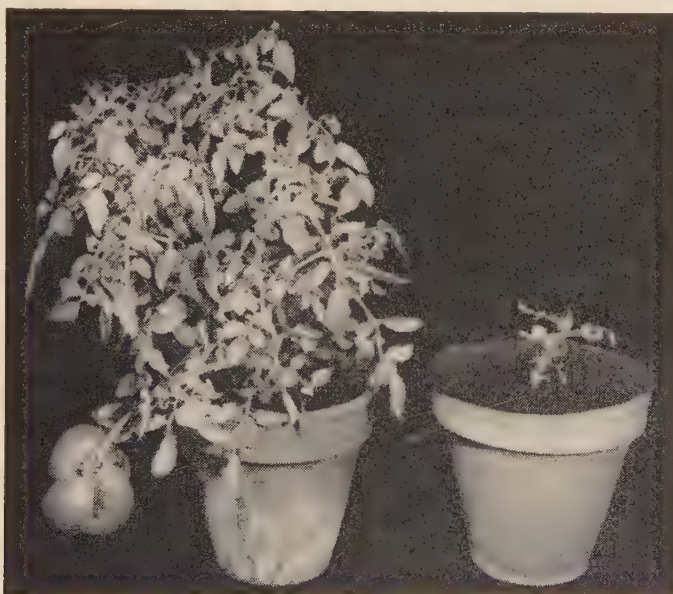


Fig. 1.—Plants homozygous recessive for the gene conferring thiamine requirement; age 106 days. Right, untreated control. Left, plant sprayed three times per week with thiamine at the rate of 2 mg per 100 ml.

Lindegren and Lindegren (1951). After mutant plants, which received three treatments with thiamine (2 mg per 100 ml water) per week, had been grown for about four months, they were almost indistinguishable from the wild-type. The plants were nearly normal in size, normal in chlorophyll formation, flowering, and seed production (Fig. 1); their progeny were all mutant in phenotype.

In all organisms that have been studied, thiamine formation has been shown to occur by the separate synthesis of a substituted pyrimidine and a substituted thiazole ring followed by the linking of these compounds by a methylene bridge. Therefore, mutant plants were sprayed with "pyrimidine" (2-methyl-6-amino-5-aminomethylpyrimidine) and "thiazole" (4-methyl-5- β -hydroxyethylthiazole) both separately

and together to obtain information on the biochemical deficiency. The mutant responded to "pyrimidine" but not to "thiazole" alone, indicating that the genetic block is in the formation of "pyrimidine" and that "thiazole" synthesis and the coupling reaction are unimpaired by the mutation (Fig. 2, *A*). Robbins and Bartley (1937) have shown that isolated roots of the tomato are unable to form thiamine but here the deficiency is in the ability to make the thiazole part (Fig. 2, *B*). Langridge (1958) has described an *Arabidopsis* mutant that is unable to join together the two halves of the thiamine molecule (Fig. 2, *C*).

Since a pyrimidine compound is required by the mutant, tests were made of the plants' ability to synthesize the pyrimidines of the nucleic acids. Deficient plants were sprayed with cytosine (2-hydroxy-6-aminopyrimidine), uracil (2,6-dihydropyrimidine), and thymine (5-methyl-2,6-dihydropyrimidine). All these compounds were without visible effect.

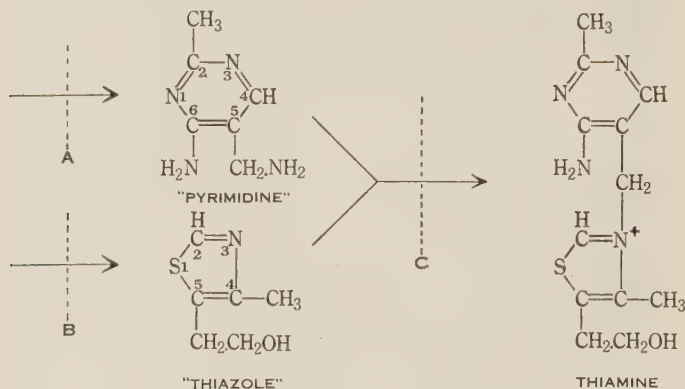


Fig. 2.—Diagram of the structure and synthesis of thiamine. *A*, location of the block in the tomato mutant. *B*, location of the block in isolated roots of the tomato. *C*, location of the block in an *Arabidopsis* mutant.

The various pyrimidines of the nucleic acids have a common precursor, orotic acid (2,4-dihydroxy-6-carboxypyrimidine), which is also considered to be the precursor of the pyrimidine portion of the thiamine molecule. For physiological activity, the pyrimidine ring of thiamine needs an amino group at position 6 (Bergel and Todd 1937), a methyl or ethyl group at position 2 (Huber 1943), and a reactive group at the 5-methyl position for bridge formation with thiazole (Bonner 1938). The absence of response to cytosine and thymine indicates that the mutant is able to perform the 5-methyl and 6-amino substitutions and to remove the hydroxyl group at position 4. Therefore, the chemical deficiency in this mutant must be either in the replacement of a hydroxyl at position 2 by a methyl group, or in the forming of a reactive group at the 5-methyl position.

As a result of the lack of thiamine, there is an almost complete absence of chlorophyll in mutant plants. The chloroplasts of the mutant are normal in size and appearance, so thiamine must be required at some stage in the formation of the chlorophyll molecule itself. However, no chlorophyll was formed by treatment of

deficient plants with δ -amino laevulinic acid, the precursor of the pyrrole ring, or by porphobilinogen, the precursor of the porphyrin structure. Therefore, thiamine deficiency must interfere with chlorophyll synthesis at a stage later than the formation of the pyrrole rings.

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ADDITIONAL RESISTANCE IN *TRITICUM VULGARE* TO *ERYSIPHE GRAMINIS TRITICI*

By A. T. PUGSLEY*

[Manuscript received July 28, 1960]

Summary

Additional genetic studies have been made of the resistance of four wheat varieties to races *P*, *P-1*, and *T* of *Erysiphe graminis tritici*.

P.I.92378, P.I.181374, and the derived variety Javelin-325 were each shown to possess the *Ulka* gene Ml_u , while Asosan was shown to possess a sixth gene, Ml_a , not previously detected.

A race of *E. graminis tritici* (race *T*) capable of attacking those varieties carrying the Ml_t gene alone provided supplementary evidence of the genetic constitution of the several experimental varieties.

Four "tester" lines, each with a Federation background, have been produced. They each carry, singly, the genes Ml_t , Ml_s , Ml_u , and Ml_a and should prove particularly useful in physiological race determinations.

I. INTRODUCTION

Genetic studies of the inheritance of resistance of 13 varieties of *Triticum vulgare* Vill. to *Erysiphe graminis tritici* have led to the identification of five genes governing the resistance of such varieties to powdery mildew (Pugsley and Carter 1953; Carter 1954). The five genes were designated Ml_t , Ml_u , Ml_s , Ml_c , and Ml_b , having been detected originally in the varieties Thew, *Ulka*, *Sonora*, *Chul*, and *Birdproof* respectively.

Mildew resistance studies have been continued over the past six years and a genetic analysis has been made of four additional varieties. The occurrence, in 1960, of a new race of *E. graminis tritici*, characterized by being able to attack those varieties carrying the Ml_t gene alone, at once provided evidence that the resistance of these four varieties was not conditioned by either of the genes Ml_t , Ml_c , Ml_s , or Ml_b . Reference to Table 1 shows that Thew (Ml_t) and *Chul*-1 (Ml_c) were susceptible and *Sonora* (Ml_s) and *Birdproof* (Ml_b) were moderately resistant to the new race.

Studies outlined in this paper were designed to secure evidence on the identity of the genes for resistance in the four varieties P.I.92378, P.I.181374, Javelin-325, and Asosan, all four being resistant to the new race *T*.

II. MATERIALS AND METHODS

Twelve varieties have already been described (Pugsley and Carter 1953). *Chul* (C.I.2227) used in Carter's (1954) studies, although uniform in its reaction to mildew, was found to be heterogeneous for auricle colour and the selection *Chul*-1, with red auricles, has been used since 1955.

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Two varieties were selected for mildew resistance from the United States Department of Agriculture world collection of varieties resistant to stem and leaf rust. They are No. 9446 (P.I.92378) from Russia and P.I.181374 from Afghanistan. The varieties C.I.12633 from the United States of America and P.I.170913 from Transvaal are believed to be derivatives of *T. timopheevi* and, although they have not been used in genetic studies, they have remained quite resistant whenever tested for mildew reaction during the past eight years.

The Japanese variety Asosan (C.I.12665) was obtained from the United States Department of Agriculture in 1954. Stevenson and Jones (1953) reported it as being resistant to mildew. Stevenson and Jones also reported Picardie (C.I.12664) from

TABLE 1

REACTION OF WHEAT VARIETIES TO RACES *P*, *P*-1, AND *T* OF *E. GRAMINIS TRITICI*

R, resistant; S, susceptible; MR, resistant but slight development of mildew on leaf sheath of seedlings

Variety	Race <i>P</i>	Race <i>P</i> -1	Race <i>T</i>	Variety	Race <i>P</i>	Race <i>P</i> -1	Race <i>T</i>
Axminster	R	R	S	Sonora	MR	MR	MR
Converse	R	R	S	Sturgeon	MR	MR	MR
Huron	R	R	S	P.I.92378	R	R	R
Kenya C.6041	R	R	S	P.I.181374	R	R	R
Norka	R	R	S	Javelin-325	R	R	R
Thew	R	R	S	Asosan	R	R	R
Birdproof	R	R	MR	C.I.12633	R	R	R
Normandie	R	R	R	P.I.170913	R	R	R
Ulka	R	R	R	Suwon 92	MR	—	—
Chul-1	S	R	S	Picardie	S	—	—
Indian	MR	MR	MR	Federation	S	S	S

France and Suwon 92 (P.I.157603) from Korea as being resistant to mildew. However, Picardie was found to be susceptible and Suwon 92 to be moderately resistant in 1955 and were not studied further.

Javelin-325 is a resistant selection of a fifth backcross, Javelin being the recurrent parent and Iumillo \times *Aegilops squarrosa* the donor parent. The amphiploid donor parent was obtained from Dr. E. P. Baker, University of Sydney, in 1951 who, in turn, had received it from Dr. E. R. Sears of Missouri, U.S.A.

During the period 1955–1959 several mildew resistant varieties were used as donor parents in a backcross programme with the standard mildew susceptible Federation as the recurrent parent. In this way several “tester” varieties have been produced each with a different gene in a common Federation background. Marker genes have been retained as an aid to their ready identification. The following tester lines are now available:

T-Federation (*Ml_t* gene), Kenya C.6041 \times Federation⁵. Marked by the *Sr₉* gene for resistance to stem rust.

S-Federation (*Ml_s* gene), Sonora \times Federation⁴. Marked by pubescent chaff.

U-Federation (Ml_u gene), Ulka \times Federation⁴. Marked by late maturity.

A-Federation (Ml_a gene), Asosan \times Federation³. Marked by white chaff and red grain.

The four tester lines together with others that may be produced in the future should prove useful as differentials in race determination studies.

TABLE 2
CLASSIFICATION OF F_2 SEGREGATES FOR THE CROSSES INDICATED WITH RESPECT TO MILDEW REACTIONS
R, resistant; S, susceptible

Cross and Parental Reactions	Resistant	Susceptible	Ratio	Value of P for Ratio Indicated
Race P, 1955				
Federation \times P.I.92378 (S \times R)	50	12	3 : 1	>0.20
Thew \times P.I.92378 (R \times R)	130	4	15 : 1	>0.10
Federation \times P.I.181374 (S \times R)	51	12	3 : 1	>0.20
Thew \times P.I.181374 (R \times R)	127	7	15 : 1	>0.50
Ulka \times P.I.181374 (R \times R)	74	0	—	—
Race $P-1$, 1956				
Asosan \times Federation (R \times S)	96	36	3 : 1	>0.50
Asosan \times Ulka (R \times R)	92	7	15 : 1	>0.70
Asosan \times Thew (R \times R)	83	7	15 : 1	>0.50
Federation \times P.I.92378 (S \times R)	117	28	3 : 1	>0.10
Thew \times P.I.92378 (R \times R)	69	5	15 : 1	>0.80
Race $P-1$, 1959				
A-Federation \times Federation (R \times S)	73	30	3 : 1	>0.30
T-Federation \times Federation (R \times S)	86	25	3 : 1	>0.50
U-Federation \times Federation (R \times S)	82	20	3 : 1	>0.20
U-Federation \times A-Federation (R \times R)	105	3	15 : 1	>0.10
U-Federation \times T-Federation (R \times R)	103	6	15 : 1	>0.70
Javelin-325 \times Federation (R \times S)	69	32	3 : 1	>0.10
Javelin-325 \times U-Federation (R \times R)	109	0	—	—
Javelin-325 \times T-Federation (R \times R)	112	6	15 : 1	>0.50
Javelin-325 \times A-Federation (R \times R)	103	7	15 : 1	>0.95
Javelin-325-Federation \times P.I.92378-Federation (R \times R)	121	0	—	—

Two tester lines, Javelin-325-Federation and P.I.92378-Federation each with the Ml_u gene were used in addition to the above in several test crosses.

Material was tested as seedlings grown in flats in the greenhouse. The races of mildew used were those which appeared in the greenhouse at the commencement of the growing season each autumn. Races P and $P-1$ (Pugsley and Carter 1953) were present during 1955 and 1956–59 respectively, while a distinctively different race (designated T) was present in 1960.

III. EXPERIMENTAL RESULTS

The reactions of the standard and experimental varieties to races *P*, *P-1*, and *T* are given in Table 1.

A limited number of segregating F_2 populations were available for testing in 1955 and 1956, the results of which are summarized in Table 2. Tentative conclusions drawn from these experiments were as follows: P.I.92378 possesses a single gene for resistance different from Ml_t carried by Thew. P.I.181374 possesses a single gene for resistance different from Ml_t but which may be identical with Ml_u carried by Ulka. Asosan possesses a single gene for resistance different from Ml_t and Ml_u . This has been designated Ml_a .

TABLE 3

CLASSIFICATION OF F_2 AND BACKCROSS SEGREGATES FOR THE CROSSES INDICATED WITH RESPECT TO MILDEW REACTIONS
R, resistant; S, susceptible

Cross and Parental Reactions	Resistant	Susceptible	Ratio	Value of <i>P</i> for Ratio Indicated
Race <i>T</i> , 1960				
Federation \times A-Federation (S \times R)	126	48	3 : 1	>0.30
Javelin-325 \times T-Federation (R \times S)	62	26	3 : 1	>0.30
Javelin-325 \times A-Federation (R \times R)	158	18	15 : 1	>0.02
Javelin-325-Federation \times U-Federation (R \times R) (U-Federation \times Javelin-325-Federation) ¹ \times Federation (R \times R) ¹ \times S	83	0	—	—
Javelin-325-Federation \times P.I.92378-Federation (R \times R)	15	0	—	—
T-Federation \times P.I.92378-Federation (S \times R)	88	0	—	—
T-Federation \times U-Federation (S \times R)	59	18	3 : 1	>0.70
T-Federation \times A-Federation (S \times R)	115	42	3 : 1	>0.50
T-Federation \times A-Federation (S \times R)	115	35	3 : 1	>0.50
(T-Federation \times A-Federation) ¹ \times Federation (S \times R) ¹ \times S	31	31	1 : 1	>0.99
U-Federation \times A-Federation (R \times R)	134	8	15 : 1	>0.70
(U-Federation \times A-Federation) ¹ \times Federation (R \times R) ¹ \times S	42	15	3 : 1	>0.80

By 1959 further segregating F_2 populations, including intercrosses between the Federation tester lines, were available, the results of which are also summarized in Table 2.

Confirmatory evidence was secured that T-Federation, U-Federation, and A-Federation carry the genes Ml_t , Ml_u , and Ml_a respectively. The results indicate that Javelin-325 possesses a single gene different from Ml_t and Ml_a but which may be identical with Ml_u of U-Federation. Failure to recover susceptible segregates from the cross P.I.92378-Federation \times Javelin-325-Federation supports the view that these two lines carry the common gene Ml_u .

The appearance of the new *T* race of *E. graminis tritici* in 1960 at once screened those varieties carrying the Ml_t gene alone—all proving susceptible (see Table 1). Two varieties, Normandie and Birdproof, previously shown to possess genes Ml_u and Ml_b , respectively, in addition to Ml_t (Pugsley and Carter 1953; Carter 1954), were resistant and moderately resistant to the *T* race.

A distinctly different pattern of behaviour was apparent when segregating populations were exposed to the *T* race, the results being summarized in Table 3. The results of several backcrosses to the susceptible Federation supported the data obtained from F_2 populations.

It should be emphasized that throughout this work the recorded results refer to the reactions of wheat seedlings to mildew. The likelihood that some type of

TABLE 4
GENES CONFERRING RESISTANCE OF WHEAT VARIETIES TO EACH OF
THE THREE RACES

Variety	Genes Conferring Resistance		
	Race <i>P</i>	Race <i>P-1</i>	Race <i>T</i>
Thew	Ml_t	Ml_t	—
Sonora	Ml_s^*	Ml_s^*	Ml_s^*
Ulka	Ml_u	Ml_u	Ml_u
Asosan	Ml_a	Ml_a	Ml_a
Normandie	Ml_t Ml_u	Ml_t Ml_u	Ml_u
Birdproof	Ml_b^* Ml_t	Ml_b^* Ml_t	Ml_b^*
Chul-1	—	Ml_c	—

* Moderate resistance only.

mature plant resistance may be operative should not be overlooked. Such appeared to be the case within the group of varieties carrying the single Ml_t gene, where Thew, Huron, and Converse were less susceptible at the heading stage compared with Norka and T-Federation.

The resistance associated with Ml_a is not completely dominant. This was observed for race *P-1* in 1956 and 1959 and, later, for race *T* in 1960. Heterozygous plants frequently, but not invariably, develop a little mildew on the leaf sheath. Heterozygous plants were always classified with the resistant group. The reaction of heterozygotes to race *P* was not determined.

IV. DISCUSSION

Of the four new varieties investigated, three were shown to possess the Ml_u gene originally detected in Ulka. The varieties are P.I.92378 from Russia, P.I.181374 from Afghanistan, and Javelin-325. The latter variety derives its resistance from the amphiploid Tumbo \times *Aegilops squarrosa*.

The fourth variety, Asosan, from Japan, was shown to possess a different gene which has been designated *MI_a*.

The Australian work has revealed the presence of six genes conditioning the resistance of *T. vulgare* to *E. graminis tritici*. As compared with this, at least 12 genes are known to confer resistance of *Hordeum vulgare* to *E. graminis hordei* (Schaller and Briggs 1954).

Four of the six genes have been incorporated in a Federation background and will be used as tester lines in future physiological race determinations.

The pattern of behaviour of all varieties to the three races of *E. graminis tritici* has been consistent over the years and in conformity with the genetic constitution proposed for each variety. The genes responsible for the resistance of seven varieties to each of the three races are listed in Table 4.

Powers, Schafer, and Caldwell (1959) have recently reported a race of *E. graminis tritici* which is pathogenic to Asosan. They refer to unpublished data of Dr. T. M. Starling which indicates that Asosan possesses a single dominant gene conditioning mildew resistance which is distinct from those described by Carter (1954).

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POSSIBLE DIFFERENTIATION IN THE WILD POPULATION OF *OENOTHERA ORGANENSIS*

By R. A. FISHER*

[Manuscript received July 26, 1960]

Summary

Two explanations, by Wright and the author respectively, have been suggested for the high number of self-sterility alleles observed in a very small wild population of *Oenothera organensis*. Wright's explanation depends on the possible differentiation due to isolation of a number of small subpopulations. Emerson's original data, however, provide a means of putting this proposal to a test, and it appears that the different subpopulations are not in fact genetically differentiated.

I. SELF-STERILITY ALLELES

In 1939 Emerson recorded his observations on the habitat and distribution of the rare species *Oenothera organensis*. The case was remarkable in that from rather few plants about 40 different self-sterility alleles were collected.

In the second edition (1958) of the "Genetical Theory of Natural Selection" I have given the solution of the problem of the distribution of the number of representations of different self-sterility allelomorphs, from which the chance of extinction may be calculated. The number of alleles observed is clearly not in equilibrium with replacement by mutation unless very high mutation rates may be postulated. It would be, however, by no means extraordinary if the species population had diminished since the last glacial period from something like 10,000 to its present small number. During this diminution many of the rarer genes would doubtless have been lost, but the rate of loss among the surviving commoner genes would be exceedingly small. Consequently, we have no right to apply the conditions of equilibrium.

Wright (1939) had earlier proposed a different solution of the problem, namely that the existing population sampled by Emerson consisted of a number of isolated populations, each maintaining its own alleles, in equilibrium with its small number, so that the aggregate of the alleles obtainable from all subdivisions could exceed the equilibrium made for an equivalent panmictic species.

Until we have clearer notions as to the nature of the process by which new alleles come into existence it would perhaps be premature to discuss this point.

Experiments on self-fertilization by Lewis (1949) have been interpreted as indicating a mutation rate for new alleles of less than 10^{-9} . However, such calculations ignore the possibility that the antibodies of the style are effective not only against the parent alleles, but against any new allele derived from them only. In any case, the proposal that there is genetic differentiation between the subpopulations sampled by Emerson can be examined from his own data.

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II. EMERSON'S TOPOGRAPHICAL DATA

From the identifications given by Emerson (1939, p. 537) it is possible to classify 31 alleles appearing as either ovule or pollen in (a) McAllister Canyon, (b) in East Fork, and (c) in North Fork, these being the principal collecting grounds. I have avoided basing conclusions on the frequencies of occurrence, since a single insect carrying pollen might have introduced the paternal gamete of a number of plants, the occurrence of which could not, therefore, be regarded as independent. If, however, there were effective isolation of the kind postulated by Wright, the occurrence of an allele in one locality would be a reason for not expecting it to occur at all in some other locality. The question is whether any tendency exists for the different localities to harbour different alleles.

TABLE I
CLASSIFICATION OF 31 ALLELES ACCORDING TO THEIR DISTRIBUTION

Locality	Number	Algebraic Expectation
McAllister's Canyon (A) only	9	$p_1q_2q_3$
East Fork (B) only	6	$q_1p_2q_3$
North Fork (C) only	3	$q_1q_2p_3$
B and C	2	$q_1p_2p_3$
C and A	5	$p_1q_2p_3$
A and B	4	$p_1p_2q_3$
A, B, and C	2	$p_1p_2p_3$
Total	31	

III. THE TEST OF INDEPENDENCE

The classification available for discussion is that of Table I. We wish to compare these numbers observed, with numbers expected, appropriate to the view that the occurrence of one allele in a locality does not affect the probability of its occurrence in another. If p_1 , p_2 , and p_3 are the probabilities of an allele chosen at random occurring in the three localities, and if in each case $p+q=1$, then the expansion of the product

$$(p_1+q_1)(p_2+q_2)(p_3+q_3)$$

will give the relative frequencies of the seven classes observed, together with an eighth unobservable class, in which the allele happens to be absent from all three localities.

To find the appropriate values of p_1 , p_2 , and p_3 we may use the device of the so-called "missing plot", and suppose that an unknown number x had been observed to be absent from all three localities. Thus in terms of x , we may set

$$\begin{array}{ll} p_1 = 20/(x+31) & q_1 = (x+11)/(x+31) \\ p_2 = 14/(x+31) & q_2 = (x+17)/(x+31) \\ p_3 = 12/(x+31) & q_3 = (x+19)/(x+31) \end{array}$$

so that we may obtain an equation for x by setting

$$q_1 q_2 q_3 (x+31) = x,$$

or

$$(x+11)(x+17)(x+19) = x(x+31)^2.$$

This leads to the quadratic equation $15x^2 + 242x - 3553 = 0$, of which the positive root is $[\sqrt{(67936) - 121}]/15$ or $139.645/15 = 9.3097$, giving

$$\begin{array}{lll} p_1 = 0.49616 & p_2 = 0.34731 & p_3 = 0.29770 \\ q_1 = 0.50384 & q_2 = 0.65269 & q_3 = 0.70230 \end{array}$$

The comparison of expected and observed frequencies is shown in Table 2.

TABLE 2
COMPARISON OF OBSERVED AND EXPECTED FREQUENCIES

Locality*	Observed ($m+x$)	Expected (m)	(x^2/m)
No locality	—	9.3096	—
A only	9	9.1677	0.0031
B only	6	4.9539	0.2209
C only	3	3.9463	0.2269
B and C	2	2.0999	0.0048
C and A	5	3.8861	0.3193
A and B	4	4.8783	0.1581
A, B, and C	2	2.0679	0.0022
Totals	31	40.3097	$0.9354 = \chi^2$

* See Table 1.

After fitting the three probabilities there are only three degrees of freedom for deviations from expectation. With χ^2 less than unity, there is no sign of any deviation beyond pure chance. With partial isolation of the alleles the expectation would be that the first three cells should appear in excess of expectation and that the last cell should be greatly deficient. Evidently, on the contrary, the frequencies observed by Emerson are indistinguishable from what would have appeared if instead of distinguishing the localities, the plants had been divided arbitrarily and by chance into three sections.

It seems likely that organisms affecting cross-pollination travelled freely over all the three localities sampled. However, if there really is isolation it has clearly led to no differentiation between the subpopulations.

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STUDIES ON THE SODIUM-POTASSIUM BALANCE IN ERYTHROCYTES OF AUSTRALIAN MERINO SHEEP

I. CHANGES IN CONCENTRATIONS IN THE ERYTHROCYTES OF LAMBS FROM BIRTH TO 98 DAYS

By JUDITH H. KOCH* and HELEN NEWTON TURNER†

[Manuscript received September 19, 1960]

Summary

Estimates are given of the sodium-potassium balance in the erythrocytes of 16 Australian Merino and Merino cross lambs at ages ranging from 0 to 98 days. The $[Na^+]$ values rose and the $[K^+]$ values fell from birth till an age of approximately 60 days, after which each variable fluctuated about a value comparable with those obtained for the dams of the same lambs, all of which were adult Merinos. Curves were fitted up to 57 days, their equations being $y[Na^+] = 21.47 + 2.63x - 0.0203x^2$ and $y[K^+] = 99.60 - 2.78x + 0.0221x^2$ (x = age in days).

I. INTRODUCTION

Evans (1954) reported differences in the sodium and potassium concentrations of the red blood cells of British breeds of sheep, which led to the recognition of two types of animal—*HK* (high potassium) and *LK* (low potassium). Evans and King (1955) concluded that the sodium-potassium balance was under genetic control and could be altered significantly by the presence of one or another of a pair of alleles at one locus, the gene for *HK* being recessive to that for *LK*. Evans and Mounib (1957) presented the results of a survey of a number of sheep breeds in Britain, which showed considerable variation in the proportion of *HK* animals in each sample of sheep. This proportion was higher for animals living in less favourable environments in the north of Britain, and the suggestion was made that the sodium-potassium balance might have some bearing on survival in mountain and upland regions in the temperate zone. Evans (1957) adapted Kerr's (1937) classification, which extended the number of recognizable types of animals from two to four, the original *LK* and *HK* now being designated *Keα* and *Keγ* respectively. Some observations on Merinos, sampled in Britain, were included in this paper, with the observation that most of them were of type *Keα*, but with an even lower value of potassium (in m-equiv/l of red blood cells) than had been recorded for other breeds. Attention was drawn to the fact that Denton *et al.* (1951) and Harris, McDonald, and Williams (1951) had also obtained very low potassium values when working with Australian Merinos, but Bernstein's (1954) values for the South African Merino were not exceptionally low.

Lamb-marking percentages are notoriously poor in the Australian Merino, frequently because of considerable losses between birth and marking, which takes

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place shortly after the end of the lambing period. In a sheep-breeding trial, one of us (H.N.T.) had observed a greater mortality in young lambs among the progeny from half-sib than from unrelated matings, and there seemed a possibility that some detrimental recessive gene might be contributing to the losses. The work of Evans and his co-authors suggested that the recessive gene for high levels of potassium might be present in Australian Merino flocks, and it was thought that, for some reason homozygous recessives might be less fitted to survive in hot, dry areas than animals with a low potassium level.

To test this hypothesis, it was originally planned to sample lambs at birth, and to compare the potassium level of those which died as lambs and those which survived. At the time, no data were available on changes in the sodium-potassium balance in lamb erythrocytes. Evans and his associates have worked with sheep 6 months of age or older, and have found (Evans 1957) that repeated sampling of the same animal at various time intervals has given values which do not differ significantly from each other. Hallman and Karvonen (1949) and Widdas (1954) showed that concentrations of potassium in foetal erythrocytes were higher than those of adults in Finnish and Welsh sheep respectively, the sodium concentrations being correspondingly lower. Widdas also showed that there was a fall in the potassium concentration (y) with foetal age (d in days), as expressed by the equation $y = 115 - 0.196d$. Green and Macaskill (1928), Groenewald (1935), and Wise *et al.* (1947) all found a downward postnatal trend in the potassium level in cattle erythrocytes. In the data presented by Green and Macaskill and by Groenewald, there was a corresponding rise in the sodium level, but Wise *et al.* found a fall in sodium level to 14 days of age, followed by a rise.

The present set of observations was undertaken to give information on changes in the sodium-potassium balance of the erythrocytes of Merino and Merino cross lambs immediately after birth. Subsequently, Wright *et al.* (1958) published observations on four lambs, at ages ranging from 14 to 63 days, no details of breed being given. The pattern was similar to that for cattle, with a rise in $[Na^+]$ and a corresponding fall in $[K^+]$.

II. MATERIAL AND METHODS

(a) Sheep

Data in this first series of observations came from Merino ewes and Merino or Merino \times Border Leicester crossbred lambs born to them at three centres in or near Sydney during September-October 1955—the McMaster Animal Health Laboratory, Glebe, N.S.W., Ian Clunies Ross Animal Research Laboratory, Prospect, N.S.W., and the F. D. McMaster Field Station, Badgery's Creek, N.S.W. Sheep at the two laboratories were maintained under pen conditions, while those at the field station were run at pasture. No differences in potassium or sodium concentrations were detected between sheep from the three locations, nor were the data sufficiently numerous to demonstrate any possible differences between sheep of different sexes, or between pure Merinos and crossbreds. All observations have therefore been considered together.

A total of 16 ewes and 21 lambs were sampled, including five sets of twins. One ewe sample was lost before measurement.

The animals were sampled as soon as possible after the birth of the lamb, and at intervals from then on. Each surviving lamb was bled from four to seven times. Samplings were fairly regular up to seven weeks of age, but thereafter only a few readings were made, the oldest lamb sampled being aged 98 days.

(b) Sampling and Measurement

The method used was based on that of Love and Burch (1953). About 5 ml of blood was collected from the jugular vein of the sheep, using an 18-gauge needle, and was run directly into a plastic centrifuge tube (see below) containing 0.5 mg of crystalline heparin (Boots) dissolved in 0.1 ml of a 0.154M NaCl solution. The tubes containing the blood were immediately placed in a centrifuge and spun at 3000 r.c.f. for 30 min. After spinning, a clamp was fastened just below the top of the red cell layer. The top part of the tube above the clamp, which contained plasma, white cell layer, and portion of the packed red cells was cut off and discarded. The clamp was then released and the remaining lower portion of the tube sealed, care being taken that the cork stopper did not touch the surface of the packed red cells. The sample was then immersed in a mixture of dry ice and alcohol, the frozen samples being later placed in the freezing chamber of a refrigerator. Immediately before analysis, the samples were thawed by placing the tubes in tap water. The resulting haemolysed solutions were diluted with double-distilled water, appropriately for the estimation of Na and K on the "EEL" flame photometer. Measurements are expressed in milli-equivalents per litre of packed red blood cells (m-equiv/l) and denoted by $[Na^+]$ and $[K^+]$ respectively.

The plastic centrifuge tubes were prepared from polythene tubing of 15 mm bore and 1 mm wall thickness. The hose was cut into pieces 10 cm long, one end of each piece then being rounded off and heat-sealed above a bunsen flame. These tubes could be used over a period of 2-3 months, after which the sealed end seemed to become brittle and reopened.

III. RESULTS AND DISCUSSION

Full data for lambs which survived till the end of the period of observations are given in Table 1, and for those which died in Table 2.

To obtain homogeneous sets of data, with as many observations as possible at each day of age, the readings shown in brackets in Table 1 were interpolated on trends fitted by eye for each lamb; in most cases linear interpolation between adjacent readings was appropriate. Using these interpolated values and grouping consecutive days, three sets of homogeneous data were obtained, with means for lambs 1-4, 5-10, and 12-16. The group of lambs 1-4, for example, has mean values for days 1-2, 5-6, 12-13, 19-20, 26-27, 40-41, and 61-62. By further interpolations of the same kind, the two Merino sets (lambs 1-4 and 5-10) were combined into one, with readings at ages ranging from day 1 to day 56/57. Two minor extrapolations were used for lambs 8 and 9, the readings for days 47 and 50 respectively being repeated at day 57. Extensive extrapolation was not considered warranted, and

the means for crossbred lambs (12-16) were kept separate, with readings at ages from day 11 to day 91.

TABLE 2

VALUES OF SODIUM AND POTASSIUM (IN M-EQUIV/L PACKED RED BLOOD CELLS) FOR LAMBS WHICH DIED DURING THE PERIOD OF OBSERVATIONS

Age (days)	Merino Lambs				Crossbred Lambs			
	No. 17		No. 18		No. 19		No. 20	
	[Na ⁺]	[K ⁺]	[Na ⁺]	[K ⁺]	[Na ⁺]	[K ⁺]	[Na ⁺]	[K ⁺]
0	22	88	26	110	23	96	23	103
4								
7	52	84						
27	81	51						
50	120	15						

The means of [Na⁺] and [K⁺] for the 10 Merino and 5 crossbred lambs are plotted on Figure 1. Individual values lying outside the range of days used for

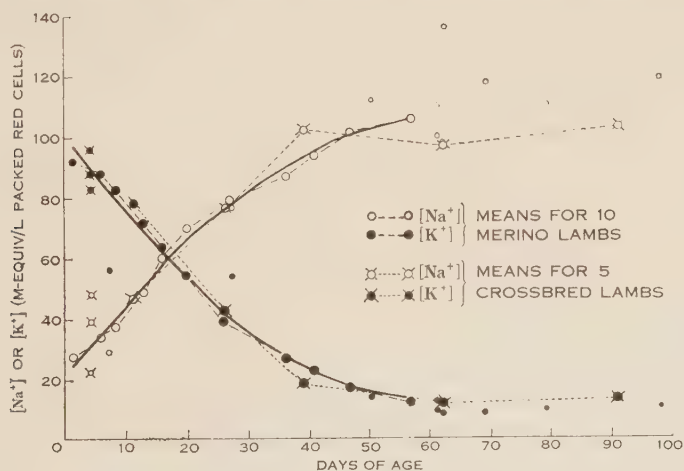


Fig. 1.—Changes with age of [Na⁺] and [K⁺]. Individual readings not included in the derivation of the means (see text) are shown in the same code with smaller symbols. Full lines indicate the curves fitted to the means of the 10 Merino lambs up to 56/57 days, the equations being

$$y[\text{Na}^+] = 21.47 + 2.63x - 0.0203x^2,$$

$$y[\text{K}^+] = 99.60 - 2.78x + 0.0221x^2.$$

the means are shown separately, together with the readings for Merino lamb 11, which had no readings before day 7.

From Figure 1 it is clear that the [Na⁺] values rise with age, while the [K⁺] values fall, until each is fluctuating about a relatively constant level. The exact

point at which this level is reached cannot, of course, be determined, and is likely to vary from sheep to sheep. In the present data there is clearly no further trend in either variable after 60 days, and very little trend after 50 days. Beyond 60 days the $[\text{Na}^+]$ values appear to fluctuate more than those for $[\text{K}^+]$, but as only a few figures are available no definite conclusion can be drawn on this point.

Quadratic curves were fitted to the means for the 10 Merino lambs, the equations being:

$$\begin{aligned}y[\text{Na}^+] &= 21.47 + 2.63x - 0.0203x^2, \\y[\text{K}^+] &= 99.60 - 2.78x + 0.0221x^2,\end{aligned}$$

where x = age in days. The curves have been plotted on Figure 1 up to $x = 60$. The fit is good; there is a suggestion of a sigmoidal shape in each case, but observations at early ages are not sufficiently close for the more refined curve to be fitted accurately. Variances about the fitted lines, calculated from the deviations of observed values, were similar, the standard errors of the regression coefficients being 0.75 for the linear and 0.013 for the quadratic terms.

The two curves are thus reversed, the two pairs of coefficients being opposite in sign but not differing significantly in magnitude. The sum of $[\text{Na}^+]$ and $[\text{K}^+]$ remains relatively constant, but there is a slight tendency for a downward trend with age. This can be seen by comparing the mean sums at earlier and later ages for the same sheep; for example, the weighted mean difference between the mean sum before and after 20 days of age (chosen as having approximately the same number of observations before and after) was -3.0 for lambs 1–10, the change being from 121 to 118. This trend is reflected in the fact that the negative linear regression coefficient for $[\text{K}^+]$ is greater than the corresponding positive coefficient for $[\text{Na}^+]$. The difference in magnitude was not significant, and neither was the linear coefficient fitted to $[\text{Na}^+] + [\text{K}^+]$ whose value was -0.103 ± 0.222 . The tendency towards a downward trend in the sum is, however, worth noting.

The mean values for the five crossbred lambs follow the general pattern for the Merino lambs, as do the additional individual values (Fig. 1), though there are occasional aberrant observations.

It is interesting to compare the rates of change in $[\text{K}^+]$ with those given by Widdas (1954) for foetal lambs. Widdas found a negative linear trend of $[\text{K}^+]$ on foetal age, the fall being at the rate of -0.196 m-equiv/l per day of foetal age. If the quadratic term is omitted, the linear trend for $[\text{K}^+]$ in the fitted line of Figure 1 is -1.605 , which is 8 times the foetal value found by Widdas. As mentioned previously, Figure 1 gives an indication that the fall in $[\text{K}^+]$ is at a lower rate during the first few days of life. The estimated combined means for day 1 and day 5.5 for lambs 1–10 were 92.8 and 88.0 m-equiv/l respectively, giving a daily fall of -0.873 , which is still 4.5 times Widdas' figure.

The mean value of $[\text{Na}^+]$ from 60 days onward is 105.8, including all values, the range being from 74.0 to 137.0 m-equiv/l. The mean value of $[\text{K}^+]$ from 60 days onwards is 11.0, the range being from 8.3 to 16.0. Both ranges are comparable with the values obtained for the adult ewes in this experiment, which had an average for $[\text{Na}^+]$ of 97.4 (range 88–114) and for $[\text{K}^+]$ of 11.5 (range 9–22). It would therefore

appear that the adult value for both criteria has been reached on the average by 60 days of age, though in some cases it may be attained earlier.

The original aim of this work was to investigate the possibility of distinguishing $[K^+]$ values at birth or early in life between lambs which died and those which survived. The number of deaths (five) in the present group was small. Table 2 shows readings at birth for four of them; the $[K^+]$ values range from 88 to 110, compared with a range of 84 to 115 for readings at birth or 1 day old for eight lambs which survived. (Table 1). The ranges overlap completely, and the numbers are too small for any conclusions to be drawn about differences between dead and surviving animals. All $[K^+]$ values at birth appear to be so high, however, that the chance of demonstrating any difference is very slight.

A new series of observations was therefore planned, estimates being made on adult ewes and rams at mating, with a view to investigating the possibility of an association between the $[K^+]$ values of the parents and the outcome of the mating.

IV. ACKNOWLEDGMENTS

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ELECTROLYTE AND HAEMATOCRIT CHANGES IN THE BLOOD OF SHEEP FROM FOETAL TO POSTNATAL LIFE

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Summary

Foetal and postnatal packed cell volume percentages and whole blood, plasma, and erythrocyte potassium and sodium concentrations have been studied in the sheep.

A significant increase in packed cell volume and whole blood potassium was found between 70 and 140 days postconception.

A significant decrease in erythrocyte potassium concentration occurred over the same period. The equation

$$Y = 64.23 + (1.017x - 0.0059x^2) \text{ m-equiv/l,}$$

where x = foetal age in days, best fits the data.

Postnatal data fits well with prenatal data and a decrease in packed cell volume, whole blood potassium, and erythrocyte potassium associated with an increase in whole blood and erythrocyte sodium was recorded over the first 105 days of postnatal life. The red blood cell potassium concentration fell from 91.4 to 15.5 m-equiv/l over this period.

The results are discussed in relation to previous work and the possibility of a change over from one type of cell to another over the period in which the blood factors were studied.

I. INTRODUCTION

Differences in the concentration of potassium between foetal and adult erythrocytes have been shown for several species. Green and Macaskill (1928) reported that whole blood from calves had higher concentrations of potassium than blood from adult cows. Hallman and Karvonen (1949) noted similar differences in the whole blood of sheep, and demonstrated that these differences were related to differences in the concentrations of potassium in the erythrocyte. A fall in the concentration of potassium in the calf erythrocytes was shown to occur during the first 90 days of postnatal life by Wise *et al.* (1947) and in the lamb by Wright *et al.* (1958). A decrease with age in the concentration of potassium in the erythrocytes of foetal lambs has been shown by Widdas (1954), the relationship being represented by the equation $K = (115 - 0.196d)$ m-equiv/l, where d is the foetal age in days. The reverse situation in man and pig has been reported by McCance and Widdowson (1956) who showed that concentrations of potassium in the erythrocytes of these species are lower in the foetus than the adult.

The concentration of potassium in the erythrocytes of adult man and pig is of the order of 100 m-equiv/l, and no evidence of marked variation from this value in normal animals has been reported. Adult sheep can be divided, however, into two

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groups according to the concentrations of sodium and potassium in their erythrocytes (Kerr 1937; Hallman and Karvonen 1949; Evans 1954). Any individual has red blood cells which are either of the low potassium/high sodium type or of the high potassium/low sodium type. A similar bimodality was found to exist in the erythrocytes of the goat, but cattle were found to be uniformly of the low potassium/high sodium type by Evans and Phillipson (1957). Recently evidence for high potassium erythrocytes in cattle has been found (Evans, unpublished data). These two types in the sheep have been designated *LK* and *HK* respectively (Evans 1954). This difference in the electrolyte composition of erythrocytes has been shown to be a permanent characteristic (Evans 1957), and to be genetically determined in a simple Mendelian manner, *HK* being recessive (Evans and King 1955; Evans *et al.* 1956). Most breeds are polymorphic for these characters (Evans and Mounib 1957; Evans, Harris, and Warren 1958).

Widdas (1954), who was using foetuses from *HK* and *LK* Welsh mountain ewes, fitted a single linear regression to his data. This implies that the difference between the genotypes is only expressed after birth and that the postnatal fall in the concentration of potassium in the erythrocyte of *HK* and *LK* lambs must be at different rates.

It is difficult to imagine that the expression of the genotype controlling the concentration of potassium in the red blood cells of sheep is absolutely dependent upon extra-uterine life particularly when it is known that adult haemoglobin is initially synthesized no later than the last third of pregnancy and that at birth 20–50% of the circulating haemoglobin is of the adult type (Karvonen 1949; Barron 1951). It would be most interesting, however, if the gene did express itself only after pulmonary respiration had commenced.

If the blood of the foetuses which are potentially *LK* animals was to be examined over the last 70 days of pregnancy any tendency for a greater rate of decrease in the concentration of potassium in the red blood cells over the last 4 weeks should be demonstrable and it should be possible to relate it to the fall which is known to occur in *LK* lambs from birth to 4 months of age (Wright *et al.* 1958).

The experiment reported here was an attempt to study these relationships.

Unfortunately, *HK* animals are uncommon in most of the breeds of sheep which are readily available in Australia (gene frequency for *HK* in Merino = approx. 0.07 (Evans 1960)) and it was not possible therefore to compare the rate of decrease of potassium in the red blood cells of potential *HK* with that in potential *LK* animals. This will be done when suitable flocks have been established.

II. MATERIALS AND METHODS

(a) Materials

(i) *Prenatal*.—Foetal blood was collected from the progeny of an *HK* Southdown ram and *LK* Romney ewes which had been slaughtered at 10-day intervals from the 70th–140th day of gestation. Altogether 26 foetuses from 24 ewes were examined. Blood was expressed from the umbilical vessels and collected into 30-ml heparinized screw-cap bottles which were both sodium- and potassium-free.

(ii) *Postnatal*.—Six Merino lambs, the progeny of *LK* Merino ewes and an *HK* Merino ram, were used. Blood samples were taken within 24 hr of birth and again at 3-weekly intervals for a period of 15 weeks. 10 ml of blood was withdrawn from the jugular vein on each occasion into a sodium- and potassium-free heparinized syringe.

(b) *Methods*

Plasma samples were obtained by centrifugation at 1750 *g* for 30 min. Both plasma and whole blood samples were diluted 1:100 with deionized water and potassium and sodium estimations carried out on an "EEL" flame photometer by the method of King and Wootton (1956). Red blood cell values were calculated from these data and the haematocrit. This method has been shown to give values equivalent to those obtained by a direct analysis of red blood cells (Barker 1958).

TABLE 1

MEAN POTASSIUM AND SODIUM CONCENTRATIONS IN WHOLE BLOOD AND ERYTHROCYTES AND PACKED CELL VOLUME IN BLOOD OF LAMBS FROM 70–140 DAYS POSTCONCEPTION

Days of Pregnancy	No. of Animals	Packed Cell Volume (%)	Whole Blood Potassium (m-equiv/l)	Erythrocyte Potassium (m-equiv/l)	Whole Blood Sodium (m-equiv/l)	Erythrocyte Sodium (m-equiv/l)
70	2	17.7±2.1	22.5±0.4	110.0±10.3	—	—
80	3	25.7±3.0	30.0±5.6	104.9±10.9	—	—
90	3	31.7±3.7	36.4±4.8	106.0±4.7	—	—
100	2	40.0±5.2	47.5±2.7	111.4±6.5	87.8*	17.3*
110	7	43.0±6.8	48.2±8.9	104.8±6.7	73.8±11.9†	34.7±11.3†
120	3	48.6±7.2	52.9±8.0	102.7±2.4	78.9*	17.1*
130	3	48.8±2.6	49.4±5.7	95.5±9.4	71.1±7.1	25.2±4.3
140	3	52.2±3.5	50.2±5.1	91.2±7.2	76.3±0.5	37.9±3.2

* Only one animal examined.

† Only three animals examined.

Haematocrit determinations on the 70- and 80-day foetal blood samples were carried out using microhaematocrit tubes. All other samples were centrifuged in Wintrobe tubes for 1 hr at 1750 *g*. Three tubes were used for each sample.

III. RESULTS

(a) *Prenatal*

The results of the examination of foetal blood from fetuses 70–140 days post-conception are shown in Table 1.

The foetal growth curve over the period during which the blood examinations were made was normal and is shown in Figure 1.

The packed cell volume increased significantly from a mean of 17.7% at 70 days to a mean of 52.2% at 140 days (Fig. 2). Linear and quadratic relationships were fitted to these data and both were significant at the 0.1% level. The difference between them is significant at the 5% level.

The concentration of potassium in the whole blood increased over the same period from a mean of 22.5 m-equiv/l to 50.2 m-equiv/l (Fig. 3). Linear and quadratic relationships were fitted to these data and both were significant at the 0.1% level but the quadratic accounted for more of the variation and was significantly different to the linear at the 1.0% level.

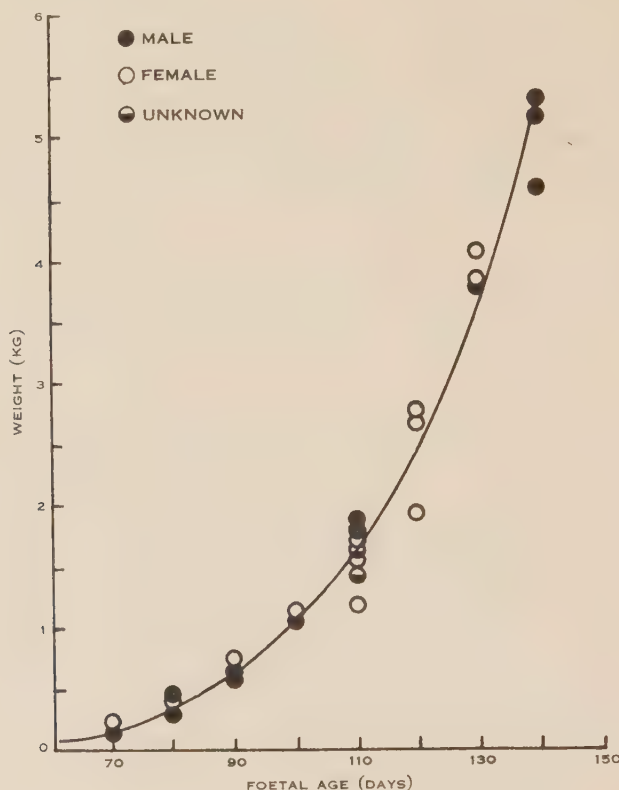


Fig. 1.—Foetal weights from 70–140 days postconception.

The concentration of potassium in the red blood cells decreased from 110.0 to 91.2 m-equiv/l over the 70-day period (Fig. 4). The linear relationship demonstrating the decrease is significant at the 1.0% level. This regression coefficient does not differ significantly from the relationship obtained by Widdas and can be expressed by the equation:

$$Y = 127.59 - (0.23 \pm 0.069)x \text{ m-equiv/l,}$$

where x is the foetal age in days. A better fit is given by a quadratic relationship, however, and the curvature is significant at the 1.0% level. The variation accounted for by the curve is 57.0% of the total, and this is significant at the 0.1% level. The equation expressing the relationship is

$$Y = 64.23 + (1.017x - 0.0059x^2) \text{ m-equiv/l,}$$

where x is the foetal age in days.

The foetal data was examined for sex differences. There was no significant difference between male and female in the weights of the foetuses or between the means in the three blood parameters examined. The females showed more scatter, however, and this was significant at the 5% level in both packed cell volume and whole blood potassium concentrations. There were significant trends, at the 0.1% level, for both sexes in packed cell volume and whole blood potassium, but only in the male whole blood potassium data was the difference between the quadratic and linear relationships significant and then only at the 5% level.

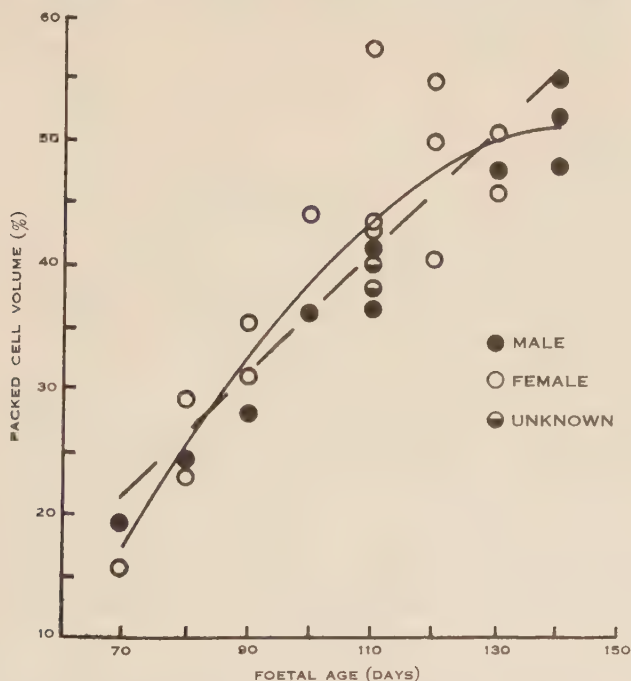


Fig. 2.—Packed cell volume (%) of the blood of foetal lambs from 70–140 days postconception.

(b) Postnatal

These data are shown in Table 2. The packed cell volume decreased from 56.0% at birth to 35.8% at 42 days and then rose to 40.0%. The concentration of potassium in whole blood also decreased from an initial value of 54.7 to 10.1 m-equiv/l at 15 weeks, while the red blood cell potassium fell from 91.4 m-equiv/l at birth to 15.5 m-equiv/l at 105 days (Fig. 5), and intracellular sodium rose from 34.9 to 87.2 m-equiv/l. The concentrations of potassium and sodium in plasma showed no significant trends during the first 15 weeks of life (Fig. 6).

Although the concentrations of potassium in the red blood cells in the foetus were higher than those obtained by Widdas (1954), the mean values at 140 days gestation and at birth in the experiments reported here are in very close agreement. This is also true of the other parameters examined in foetal and postnatal blood.

IV. DISCUSSION

Barcroft (1946) has described the relationship of blood volume to foetal age in the sheep by demonstrating that from the 11th week of pregnancy onwards the volume in the cotyledons was almost constant whereas the volume in the foetus increased approximately in proportion to foetal age. The weights of the foetuses examined in this experiment are shown in Figure 1 and demonstrate that the changes described in this paper occurred against a background of normal foetal growth. It has been shown that the mean corpuscular volume and the mean corpuscular diameter

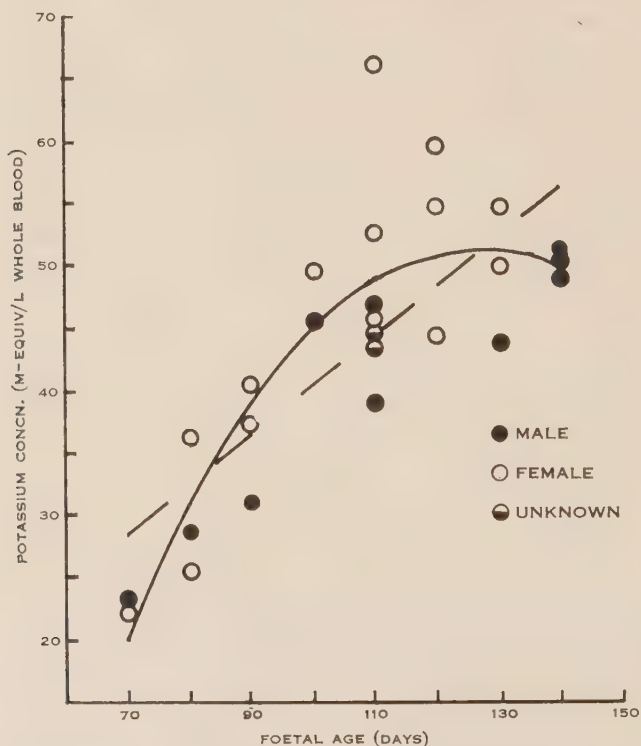


Fig. 3.—Concentrations of potassium (m-equiv/l) in whole blood of foetal lambs from 70–140 days postconception.

of foetal erythrocytes decreases with advancing foetal age in pig and man, and that the erythrocyte count increases over the same period (Wintrobe 1946). In the sheep, erythrocytes have been shown to decrease in size with advancing foetal age (Karvonen 1954). The increase in packed cell volume obtained in this experiment and illustrated in Figure 2 is most probably due therefore to a relative increase in the number of circulating cells. The increase in the concentration of potassium in whole blood (Fig. 3) tends to confirm this because the concentration of potassium in the plasma and red blood cells is relatively constant between 70 and 100 days post-conception. The foetal concentrations of potassium in the red blood cells show considerable scatter but as the linear regression for the total data for potassium concen-

trations in red blood cells is almost identical with that given by Widdas (1954) and the variation accounted for by the quadratic is significant at the 0.1% level and the quadratic gives a curve which fits the postnatal data, it seems likely that the potassium concentration in the foetal erythrocytes of potential *LK* sheep falls more rapidly in the last month of pregnancy.

Widdas (1954) was using foetuses from Welsh Mountain ewes, a breed with a gene frequency for *HK* of approximately 0.54 (Evans, Harris, and Warren 1958). Although a single linear relationship was fitted by Widdas to his data it is likely that at least 20-30% of the foetuses examined by him were potential *HK* animals. As the

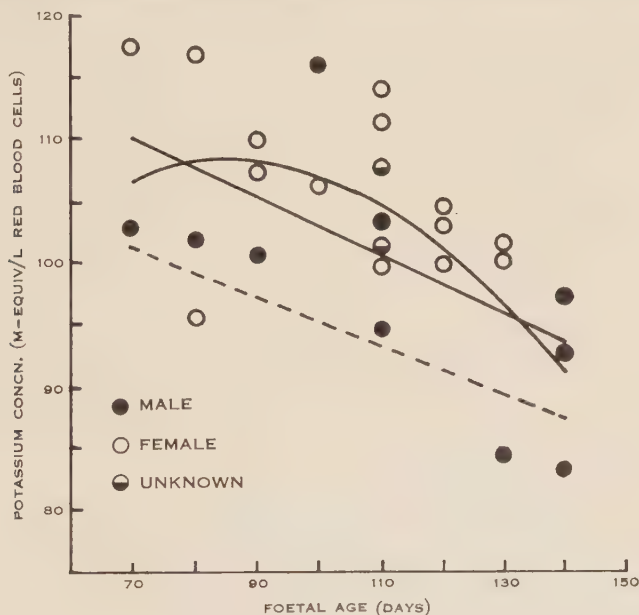


Fig. 4.—Concentrations of potassium (m-equiv/l) in erythrocytes of foetal lambs from 70–140 days postconception. ——— Least squares linear or quadratic fitted relationship. - - - Linear fitted relationship (Widdas 1954).

foetuses in the present data were from Romney ewes, none of which was *HK*, the highest percentage of potential *HK* lambs among those examined would be about 10%. Since the concentrations of potassium in the red blood cells showed a more rapid decrease after 100 days in the experiments reported here, it is suggested that the difference between *HK* and *LK* animals may be evident prior to birth.

All the lambs which were examined postnatally developed into *LK* sheep, and gave a mean concentration of potassium in the erythrocyte of 15.5 m-equiv/l at 105 days. The concentrations of potassium at birth correspond closely with those in the foetus at 140 days (Figs. 5 and 6), and the general relationships of the curves agree. The concentrations of potassium which were recorded are higher and the concentrations of sodium lower than those described by Wright *et al.* (1958). These authors found values of 5 and 6 m-equiv. potassium per litre of red cells in two lambs at 42

TABLE 2
 MEAN POTASSIUM AND SODIUM CONCENTRATIONS IN WHOLE BLOOD, PLASMA, AND ERYTHROCYTES AND PACKED CELL VOLUME IN BLOOD OF LAMBS FROM
 BIRTH TO 15 WEEKS

Six animals were examined for each criterion at each age, except at age 105 days when five animals were examined

Age (days)	Packed Cell Volume (%)	Potassium Concentration (m-equiv/l)			Sodium Concentration (m-equiv/l)		
		Whole Blood	Plasma	Erythrocytes	Whole Blood	Plasma	Erythrocytes
Birth	56.0 ± 9.4	54.7 ± 7.8	5.0 ± 0.7	91.4 ± 6.7	85.4 ± 11.9	147.8 ± 7.2	34.9 ± 17.0
21	41.9 ± 2.8	27.1 ± 3.9	4.7 ± 0.4	58.2 ± 9.7	106.6 ± 6.0	148.5 ± 4.0	49.0 ± 11.6
42	35.8 ± 3.5	13.7 ± 1.8	4.8 ± 0.4	29.9 ± 8.2	121.9 ± 0.9	136.6 ± 3.7	72.1 ± 10.8
63	38.7 ± 3.9	11.5 ± 1.0	5.3 ± 0.4	21.0 ± 3.7	123.9 ± 2.6	147.9 ± 2.0	86.9 ± 8.9
84	40.7 ± 4.2	10.3 ± 1.0	5.4 ± 0.7	17.3 ± 2.5	120.9 ± 2.4	145.8 ± 4.2	85.2 ± 10.6
105	40.0 ± 1.5	10.1 ± 1.2	5.2 ± 0.4	15.5 ± 2.9	120.8 ± 3.3	143.0 ± 3.9	87.2 ± 5.0

days and 3 and 7 m-equiv/l in two animals at 60 days, which suggests that they were dealing with *LK* sheep whose concentrations of potassium in the erythrocytes were much lower than the mean concentrations for Merino sheep recorded by Evans (1960).

The higher concentration found in this experiment could be due to the fact that all lambs would be heterozygous *LK*.

Since completing this experiment we have been privileged to see the results of a similar experiment on the postnatal concentrations of potassium and sodium of Merino and Merino \times Border Leicester lambs obtained by Koch and Turner (1960).

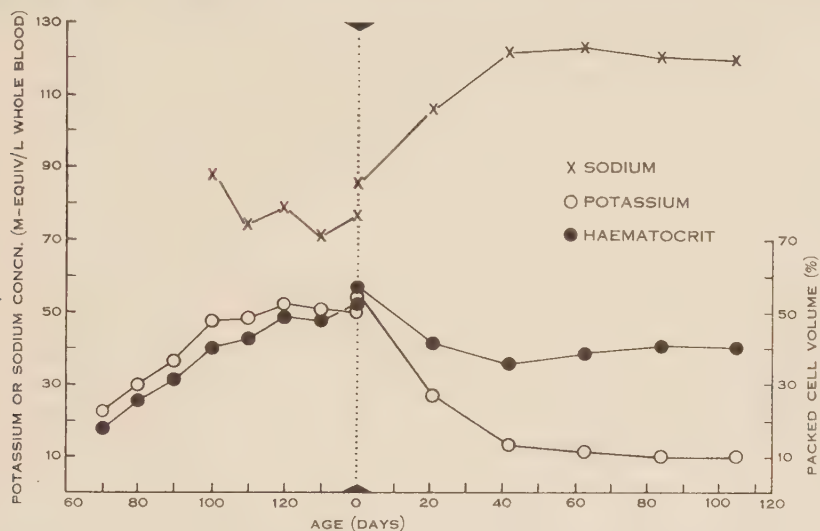


Fig. 5.—Means of whole blood concentrations of potassium and sodium (m-equiv/l whole blood) and haematocrit (%) in the erythrocytes of sheep from the 70th day postconception to 105 days after birth.

Their data shows very similar trends to those described here except that the concentrations of sodium in the red blood cells reach a peak about 10 m-equiv/l higher than recorded here at 40 days after birth and their concentrations of potassium are steady at 40–60 days at 10 m-equiv/l whereas those reported here only fall to 15.5 m-equiv/l. The differences in the final concentrations of potassium could be related to differences between animals; our animals being peculiar in that they are all heterozygous *LK*.

Similar haematocrit changes over the first few weeks of life have been reported in pigs and man by Wintrobe (1946). Wise *et al.* (1947) showed a decrease in the haematocrit of calves over the first 3 weeks of life which was followed by a rise. The sheep in this experiment (Fig. 6) showed a gradual fall up to 6 weeks of age and this was followed by a rise to a mean of 40%. This fall is probably an expression of the physiological anaemia described in other animals over the period when the growth rate of the body exceeds the rate of production of red blood cells.

Alterations in the degree of anoxaemia and the theory of a double cell population are the two hypotheses which have been evoked to explain the blood changes

which occur at birth. Karvonen (1954) has demonstrated two cell sizes in the blood of foetal sheep using Price-Jones curves, but he could not accept the interpretation of a two-cell population because the transition did not reflect the change from foetal to adult haemoglobin, nor did the development of medullary haematopoiesis fit the same time scale. He suggests that the more likely explanation for the change is the difference in oxygenation. McCance and Widdowson (1956), working with pig and man, stress the difficulty of assuming that the mechanism which extrudes sodium, matures less rapidly than the one which takes up potassium because the reverse would have to be the case in the sheep.

Various authors, who have examined foetal and adult blood, have suggested that the cell population is not homogeneous (Widdas 1955; Goodwin 1956; Kutas

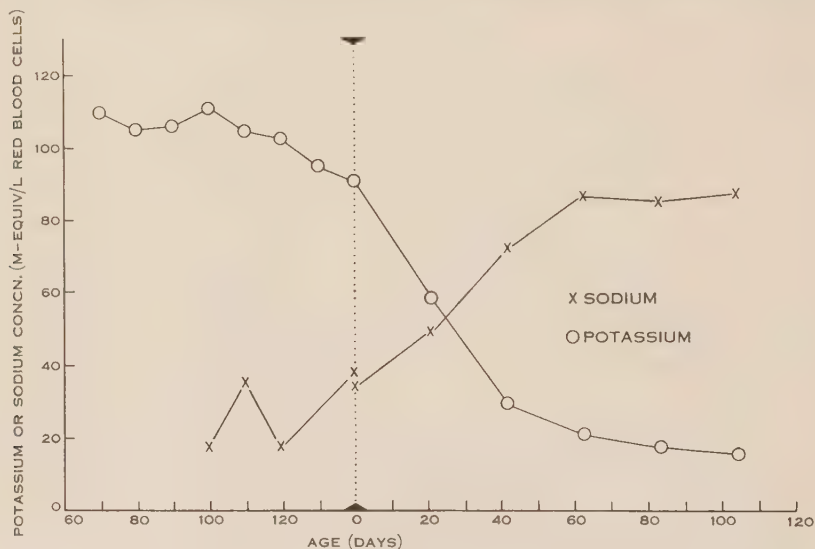


Fig. 6.—Means of the concentrations of potassium and sodium (m-equiv/l red blood cells) in the erythrocytes of sheep from the 70th day postconception to 105 days after birth.

and Stutzel 1957; Maizels 1958). Prankerd (1958) and Joyce (1958) have shown differences between young and old cells in their concentration of potassium and lipid content. Tucker (1958) has demonstrated that *HK* cells when transfused into *LK* sheep remain *HK*.

Although the difference between the oxygen dissociation curve of foetal and adult haemoglobins appears to be the result of the cell membrane rather than the type of haemoglobin (Haurowitz 1935; Hill and Wolvekamp 1936; McCarthy 1943), differences in the molecular structure of the haemoglobins have nevertheless been demonstrated (Kendrew and Perutz 1948). It is difficult to assume that one erythrocyte would contain both types of haemoglobin.

The percentage of foetal haemoglobin decreases up to birth in the human (Walker 1954) and in the sheep (Karvonen 1949; Barron 1951), and there is a more rapid decrease after birth in the human with negligible amounts of circulating foetal

haemoglobin at 22 weeks (Jonxis 1949). These data give a curve which is similar to, but not identical with, the fall in potassium concentration in the present experiment, and suggest that a similar mechanism might be operating.

It can be shown that a change from one population of cells containing certain proportions of sodium and potassium to another population containing a different proportion can produce the type of changes in sodium and potassium concentrations observed in this experiment. If one assumes that one cell population contains 110 m-equiv/l of potassium and 30 m-equiv/l of sodium (foetal cells), and that another (the adult cell) contains 15 m-equiv/l of potassium and 90 m-equiv/l of sodium, and also that the rate of loss of foetal cells is equal to the rate of gain of adult cells, the proportions of foetal cells necessary to produce the concentrations of sodium and potassium described in this experiment, while adult cells are increasing, gives a curve almost identical with that of the decrease in potassium concentration which was found. While this does not prove an hypothesis it demonstrates that these changes could be brought about by alterations in cell populations.

The results obtained in this experiment enable an hypothesis to be erected which envisages that changes of red blood cell sodium and potassium could follow from two populations of cells of different composition, one of which is decreasing while the other is increasing. The results do not, however, do more than this.

Walker (1954) has suggested that the production of adult type haemoglobin and adult erythrocytes in the human is a function of age, and that foetal haemoglobin and red blood cells are produced at the low oxygen tensions of foetal life, and that when oxygen tension falls in late pregnancy foetal haemoglobin and red blood cell production is stimulated only to fail at the oxygen tensions of extra-uterine life. Foetal-type haemoglobin is known to be produced in adults in sickle cell anaemia, thalassaemia, and in acquired anaemias (Itano 1953). If the high concentration of potassium in the circulating red blood cells of the foetus is due solely to a high percentage of foetal type cells, and if foetal-type cells increase in the sheep, as in the human, towards the end of pregnancy one would expect a rise in potassium just prior to birth. This rise was not recorded in this experiment and the fall from 100 days postconception which was obtained suggests that changes in oxygen tension if they do occur in the sheep just prior to parturition are not associated with a rise in the concentration of potassium in the red blood cells.

The number of animals examined is, however, too small to allow valid conclusions and further experiments are planned to investigate these aspects of foetal haematology.

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VARIATION IN THE GENE FREQUENCIES OF POTASSIUM AND HAEMOGLOBIN TYPES IN ROMNEY MARSH AND SOUTHDOWN SHEEP ESTABLISHED AWAY FROM THEIR NATIVE ENVIRONMENT

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Summary

The gene frequencies for high potassium and haemoglobin type *A* have been determined in flocks of Romney Marsh and Southdown sheep in New South Wales and these frequencies have been compared with those found in Great Britain.

The high potassium gene frequency in the New South Wales flocks which were examined was significantly lower in both the Romney Marsh and Southdown breeds compared with the same breeds in Great Britain.

The haemoglobin *A* gene frequency in the Romney Marsh was significantly higher than that found in Great Britain but the Southdown flocks showed the reverse tendency. However, when Down breed or Shortwool breed gene frequencies from Great Britain were compared with the Southdown results in New South Wales, a similar and significant change to that seen in the Romney Marsh was demonstrated.

All changes were toward the normal frequencies in Merino sheep, and the possible economic significance of this is discussed.

I. INTRODUCTION

Sheep can be classified into two types—low potassium (*LK*) and high potassium (*HK*)—with respect to the concentration of potassium in their red blood cells (Evans 1954). The two types are simply inherited (Evans and King 1955), and the gene frequency for *HK* varies from breed to breed (Evans 1954; Evans, Harris, and Warren 1958*a*, 1958*b*). A bimodal distribution of potassium concentrations in the erythrocytes of different animals within a species has only been recorded in the sheep (Evans 1954; Evans, Harris, and Warren 1958*a*, 1958*b*), the goat (Evans and Phillipson 1957), the Australian possum (Barker 1958), and the ox (Evans, unpublished data).

In sheep two types of haemoglobins have been separated electrophoretically and named haemoglobin *A* and *B* respectively (Harris and Warren 1955) and the inheritance of the two types has been worked out (Evans *et al.* 1956).

The genes associated with potassium types and haemoglobin types have been shown to be at different chromosomal loci (Evans *et al.* 1956) but a correlation between these two factors between breeds is present (Evans, Harris, and Warren 1958*a*).

The Merino has a very low gene frequency for *HK* (*c.* 0.07) and a gene frequency of approximately 0.45 for haemoglobin *A*. Differences between strains of Merino exist (Evans 1960). Evans and Mounib (1957) suggested an adaptive significance for the potassium types in sheep and have shown that the British Lowland breeds have a lower gene frequency for *HK* than the Hill breeds. Evans, Harris, and Warren (1958*b*) have shown that northern European breeds are almost wholly *HK*.

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The frequencies of potassium and haemoglobin types in Romney Marsh and Southdown sheep in Great Britain have been determined (Evans, Harris, and Warren 1958*a*).

TABLE 1

NUMBER, AGE, AND SEX OF SHEEP EXAMINED, AND THE NUMBER OF EWES MATED AND RAMS USED, IN FLOCKS OF ROMNEY MARSH AND SOUTHDOWN SHEEP FROM WHICH BLOOD SAMPLES WERE OBTAINED FOR ERYTHROCYTE POTASSIUM AND HAEMOGLOBIN TYPE ESTIMATION

Flock	No. of Sheep Examined and Sex		Age (months)	No. of Ewes Mated 1958	No. of Rams Used 1958
	Potassium Estimation	Haemoglobin Estimation			
Romney Marsh					
A	48 ♀	48 ♀	9-12	714	19
B	50 ♀	49 ♀	Mixed	110	3
C	62 ♂	61 ♂	9-12	2340	54
D	52 ♀	52 ♀	9-12	260	6
E	56 ♀	53 ♀	9-12	202	8
F	53 ♀	40 ♀	9-12	273	6
G	57 ♀	57 ♀	9-12	1400	19
H	55 ♀	54 ♀	9-12	332	10
I	42 ♀	42 ♀	Mixed	—	—
J	47 ♀	—	Mixed	—	—
Totals	522	456			
Southdown					
K	54 ♀	52 ♀	9-12	212	4
L	54 ♂, ♀	54 ♂, ♀	Mixed	304	4
M	49 ♀	49 ♀	9-12	1451	29
N	32 ♀	26 ♀	9-12	59	2
O	50 ♀	50 ♀	7-10	200	5
P	65 ♀	65 ♀	9-12	454	14
Q	100 ♂, ♀	43 ♂, ♀	9-12	—	—
Totals	404	339			

It was considered possible that the very different environmental conditions between Great Britain and Australia might cause a change in the gene frequencies for *HK* and haemoglobin *A* in British breeds of sheep that had been established in Australia towards the frequencies of these genes as found in the Merino, since this latter breed seems eminently suited to the Australian environment. If a change of this nature could be demonstrated it was considered that an adaptive significance of economic importance must be postulated for these genes.

The object of this survey was to determine the gene frequencies for *HK* and haemoglobin *A* in the Romney Marsh and Southdown breeds as found in New South Wales and to compare them with those found in Great Britain.

TABLE 2
FREQUENCY OF POTASSIUM TYPES IN ROMNEY AND SOUTHDOWN FLOCKS EXAMINED, WITH ESTIMATED FREQUENCY FOR THE *HK* GENE

Flock	No. of Sheep examined	No. High Potassium Types	No. Low Potassium Types	High Potassium Types (%)	<i>HK</i> Gene Frequency	Imported Rams Used in 1958 (%)	Imported and Sons of Imported Rams used in 1958 (%)
Romney Marsh							
A	48	2	46	4.17	0.20	0.0	0.0
B	50	2	48	4.00	0.20	—	—
C	62	4	58	6.50	0.25	1.9	3.7
D	52	15	37	28.80	0.54	66.7	66.7
E	56	0	56	0.00	0.00	0.0	0.0
F	53	6	47	11.30	0.34	0.0	0.0
G	57	6	51	10.50	0.32	10.5	50.0
H	55	0	55	0.00	0.00	0.0	94.7
I	42	2	40	4.76	0.22	—	0.0
J	47	0	47	0.00	0.00	—	—
Totals	522	37	485	7.09	0.27*		
Southdown							
K	54	0	54	0.00	0.00	0.0	0.0
L	54	8	46	14.8	0.38	100.0	100.0
M	49	2	47	4.1	0.20	2.9	11.4
N	32	12	20	37.5	0.61	0.0	0.0
O	50	5	45	10.0	0.32	0.0	40.0
P	65	4	61	6.2	0.25	7.1	28.6
Q	100	6	94	6.0	0.24	—	—
Totals	404	37	367	9.16	0.30†		

* *HK* gene frequency for Romney Marsh in Great Britain (Evans, Harris, and Warren 1958a) = 0.53. Difference significant at 0.1% level.

† *HK* gene frequency for Southdown in Great Britain (Evans, Harris, and Warren 1958a) = 0.44. Difference significant at 0.1% level.

II. MATERIAL AND METHODS

(a) *Material*

The sheep examined were from 10 Romney Marsh flocks and seven Southdown flocks. Eight of the Romney and six of the Southdown flocks were stud flocks on the Central and Southern Tablelands of New South Wales. The remaining two Romney flocks were mixed-age ewes from the C.S.I.R.O. Field Station, Chiswick, N.S.W., and one Southdown flock was a stud flock from the Northern Tablelands. Approximately 50 sheep were sampled from each flock.

The age of the sheep varied but they were mostly 9–12-month old ewe lambs. Mixed-age ewes were bled in three flocks and rams, aged 9–12 months, were sampled on three occasions.

The number, age, and sex of sheep bled in each flock are shown in Table 1. The 9–12 month old lambs which were sampled were the progeny of the 1958 mating; the number of ewes mated and the number of rams used in this season for each flock were obtained from the British Breeds Stud Book and are also shown in Table 1. The flocks in the districts from which most of the samples were taken are not very large.

(b) *Methods*

The sheep were collected into yards the night before bleeding. The blood was withdrawn by jugular vein puncture with the sheep in the standing position, and about 10–15 ml were collected into sterile 30-ml McCartney bottles containing 1.5 μ g heparin. The samples were dispatched to the laboratory as soon as possible after collection.

The concentrations of potassium in whole blood were estimated using an "EEL" flame photometer (King and Wootton 1956).

The haemoglobin preparations were made by centrifuging whole blood at 3000 r.p.m. for 30 min and discarding the plasma. The cells were then washed in normal saline three times and finally haemolysed in an equal quantity of distilled water. The haemolysate was subjected to electrophoresis on Whatman No. 3 MM paper in a horizontal-type bath using a Tris-borate buffer at a pH 9.1 (Tris 60.5 g, boric acid 4.6 g, final volume 1 litre). The bath was run at 225 V for 16 hr.

III. RESULTS

The number of sheep of each of the two potassium phenotypes in 10 Romney Marsh and seven Southdown flocks are shown in Table 2 together with the estimated frequency of the *HK* gene.

Table 3 shows the number of each of the three haemoglobin types found in each flock, together with the gene frequency for haemoglobin *A*, and the observed numbers are compared with the theoretically expected numbers.

Most flocks were polymorphic with respect to the potassium and haemoglobin characters and there was considerable variation between flocks. The gene frequency for *HK* in the Romneys was 0.27, compared with 0.53 in Great Britain. The range was between 0.00 and 0.54. In the Southdowns the frequency was 0.30, as against 0.44 in Great Britain, with a range of 0.00–0.61.

TABLE 3
FREQUENCY OF HAEMOGLOBIN TYPES SHOWING OBSERVED AND EXPECTED NUMBERS OF A, AB, AND B TYPES

Flock	No. of Sheep Examined	No. Observed			Type A Gene Frequency	No. Expected		
		Type A	Type AB	Type B		Type A	Type AB	Type B
Romney Marsh								
A	48	5	22	21	0.33	5.23	21.22	21.55
B	49	14	28	7	0.57	15.92	24.02	9.06
C	61	8	28	25	0.36	7.91	28.11	23.99
D	52	14	24	14	0.50	13.00	26.00	13.00
E	53	19	23	11	0.57	17.23	25.98	9.80
F	40	14	25	1	0.66	17.42	17.93	4.62
G	57	7	27	23	0.36	7.39	26.27	23.25
H	54	1	17	36	0.18	1.75	15.94	36.31
I	42	10	23	9	0.51	10.92	21.00	10.08
Totals	456	92	217	147	0.44*	96.77	206.47	151.66
Southdown								
K	52	1	24	27	0.25	3.25	19.50	29.25
L	54	2	21	31	0.23	2.86	19.13	32.02
M	49	6	23	20	0.36	6.35	22.58	20.17
N	26	0	9	17	0.17	0.75	7.34	17.91
O	50	3	35	12	0.41	8.41	24.19	17.41
P	65	4	36	25	0.34	7.51	29.17	28.31
Q	43	0	9	34	0.10	0.00	7.74	34.83
Totals	339	16	157	166	0.28†	29.13	129.65‡	179.90

* Haemoglobin type A gene frequency for Romney Marsh in Great Britain (Evans, Harris, and Warren 1958a) = 0.11. Difference significant at 0.1% level.

† Haemoglobin type A gene frequency for Southdown and Down breeds in Great Britain (Evans, Harris, and Warren 1958a) = 0.48 and 0.09 respectively. Differences significant at 1% and 0.1% levels respectively.

‡ Observed value significantly higher than expected ($P < 1.0\%$).

The frequency for haemoglobin *A* in the Romneys was 0.44 and in the Southdowns 0.28, compared with 0.11 and 0.48, respectively, in Great Britain. The variation between flocks in the Romney was 0.18–0.66, and in the Southdowns 0.17–0.41. The expected numbers of the haemoglobin types, assuming a Hardy–Weinberg equilibrium, did not agree with those observed. In each case there was an excess of heterozygotes.

IV. DISCUSSION

The frequency for the *HK* gene in the two breeds examined was considerably lower than that reported by Evans, Harris, and Warren (1958*a*) for the same breeds in Great Britain. These authors also showed that, in general, differences between breeds in Great Britain were greater than differences between flocks of the same breed, and that the frequencies in three Romney flocks was 0.47, 0.56, and 0.57. The range for the frequency of the *HK* gene in the various flocks examined in this survey was much wider than that observed in Great Britain.

The Merino has a gene frequency of approximately 0.07 for *HK* (Evans 1960) and appears to be able to exist under much hotter and drier conditions than British breeds (Wright 1954). *LK* animals have been shown in one breed (Scottish Blackface) to drink less water and pass less urine than *HK* animals (Evans 1957). It seems reasonable to associate the change towards a lower gene frequency for *HK* which was found in both the British breeds, which had been established in Australia and were examined in this survey, to a significant effect of these genes on the adaptation of these sheep to the Australian environment. It also seems reasonable to suggest that differences in water metabolism or lambing percentages may be involved (Evans 1960).

The argument is strengthened by the observation that most of the higher frequencies for *HK* seemed to be associated with flocks which used high percentages of imported rams. A positive correlation between these two factors is on the borderline of significance at the 5% level. The exception is flock N, which, however, used only two rams as the sires of the sheep examined. The imported rams were either from New Zealand or Tasmania.

Interpretation of the changes in the frequencies of the haemoglobin phenotypes is more difficult. In the Romney Marsh sheep there is a greater frequency for haemoglobin *A* compared with the same breed examined in Great Britain ($P < 0.1$), whereas the Southdowns have a lower frequency for haemoglobin *A* compared with the same breed in Great Britain ($P < 1.0$). However, Evans, Harris, and Warren (1958*a*) who only examined one Southdown flock in Britain state "The most anomalous result is that obtained with respect to the haemoglobin gene in the Southdown . . . Its position in the overall geographic pattern is anomalous". The mean gene frequency for the five Down flocks examined by them was 0.09 and for the nine Lowland Shortwool flocks 0.07, but the frequency for the Southdown was 0.48 despite the fact that the Southdown had been used in the establishment of all the other Down breeds. Evans, Harris, and Warren suggest that the Southdown haemoglobin *A* frequency obtained by them "may be due to chance fluctuations". The Southdown *HK* gene frequency was in no way anomalous, however, with respect

to other Down or Shortwool sheep. The significant increase in the gene frequency for haemoglobin *A* in the Romney Marsh in Australia compared with Great Britain and the fact that a similar significant tendency ($P < 0.1$) would be shown in the Southdown if mean Down or Lowland breed haemoglobin *A* frequencies rather than Southdown frequencies obtained in Britain were taken suggests that the Southdown flock examined by Evans, Harris, and Warren may not have been representative of the breed and that in Australia the change to a higher frequency for haemoglobin *A* in the Romney Marsh breed may be paralleled by a similar change in the Southdown.

TABLE 4

COMPARISON BETWEEN THE GENE FREQUENCIES FOR *HK* AND HAEMOGLOBIN *A* IN SHEEP IN GREAT BRITAIN AND AUSTRALIA

Breed	<i>HK</i> Gene Frequency		Haemoglobin <i>A</i> Gene Frequency	
	Great Britain	Australia	Great Britain	Australia
Romney Marsh	0.53	0.27	0.11	0.44
Southdown	0.44	0.30	0.09* 0.48†	0.28
Merino		0.07		0.45

* Down breeds excluding Southdown (Evans, Harris, and Warren 1958a).

† Southdown breeds (Evans, Harris, and Warren 1958a).

The excess of heterozygotes (haemoglobin *AB*) was significant at the 1% level in the Southdowns but not significant in the Romneys.

If the distribution of haemoglobin types expected in a Hardy-Weinberg equilibrium is compared with the observed numbers within each breed it will be seen that the greatest deviations occur in those flocks using a small number of rams. As a general rule those flocks in which 10 or more rams were used showed the highest correlation between observed and expected numbers; the only exception was flock P, which used 14 rams during the season but the 65 sheep examined in this survey were, however, only sired by 6 of these. The significant difference between expected and observed numbers in the Southdown should therefore be interpreted with caution.

It has been shown that in British breeds in Great Britain there is a correlation between those breeds having a high gene frequency for *HK* and those with a high gene frequency for haemoglobin *A* in spite of an absence of association within breeds (Evans, Harris, and Warren 1958a) and these authors suggest that *HK* and haemoglobin *A* have some relative advantage over *LK* and haemoglobin *B* in those areas where this type of flock is indigenous. An investigation of these types in Scottish Blackface sheep in a single environment, however, did not reveal any major differences (King *et al.* 1958).

If *LK* and haemoglobin *A* are of adaptive significance in Australia—and this is suggested by the frequencies of these genes in Merino sheep (gene frequency for *LK* = 0.93 approx., gene frequency for haemoglobin *A* = 0.45 approx.)—one might expect the frequencies of these genes in British breeds in Australia to tend towards the Merino frequencies. The results reported here suggest that this does occur (Table 4).

The interpretation of these data is not clear cut but it would appear likely that *LK* sheep are better adapted to the environment from which these samples were taken and that haemoglobin *A* or haemoglobin *AB* may also have advantages over the homozygous haemoglobin *B* type animal in the same environment. If these postulated advantages can be substantiated and shown to be associated with adaptation to the Australian environment (e.g. drought, parasitism, heat) the genes controlling them become of considerable economic importance in this environment. As both potassium type and haemoglobin type are simply inherited, breeding for the superior animal would be both easy and quick.

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ON THE MECHANISM OF INCORPORATION OF [^{35}S]CYSTINE INTO WOOL

By A. M. DOWNES*

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Summary

A comparison was made of the rate of incorporation of ^{35}S into the plasma proteins and the wool of a sheep after an intravenous injection of DL-[^{35}S]cystine. The cystine of wool attained a much higher specific activity than that of the plasma proteins, suggesting that cystine is not incorporated into wool by any process involving a direct modification of the plasma proteins. It is also suggested that there is a metabolic "pool" of material containing cystine associated with the wool follicles themselves which is turned over with a half-time of about 3 days.

I. INTRODUCTION

Many advances in our understanding of protein synthesis have been made as a result of isotopic tracer experiments. For example, in a recent review of the biosynthesis of milk proteins Barry (1958) stated: "Ten years ago, it was generally concluded from arterio-venous measurement that they (milk proteins) are largely formed from plasma proteins; now it is generally concluded from experiments with tracers that they are formed largely, at least, from the free amino-acids of plasma". Other workers have concluded that proteins in general are synthesized from free amino acids without significant participation of peptide or protein residues (Loftfield 1957). Thus the hypothesis put forward by Madden and Whipple (1940) that plasma proteins may possibly be converted more or less directly to other proteins in the body has had to be rejected in most cases. However, ovalbumin seems to be an exception, since evidence for the synthesis of this protein by the oviduct from derivatives of serum proteins or intermediate peptide pools has been presented (Sankar and Theis 1959). There seems to be little evidence one way or the other on this aspect of wool biosynthesis. Ryder (1959) has shown that radioactivity is present in the wool follicles of a lamb soon after an injection of DL-[^{35}S]cystine but this evidence does not rule out the possible participation of plasma proteins in keratin synthesis. Fleischer, Lietze, Walter, and Haurowitz (1959), however, have found that hair keratin in the rat is probably synthesized from amino acids or very small peptide fragments.

Much of the evidence on the biosynthesis of milk proteins was obtained by injecting labelled essential amino acids into lactating animals and comparing the specific activities of the amino acids incorporated in casein and in the plasma proteins. During the first few hours after each injection the amino acid had a much higher specific activity in the casein and β -lactoglobulin than in the plasma proteins. These results suggested that a significant part of the essential amino acids of the

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milk proteins had come from the free amino acids of blood and not from the plasma proteins. The possibility that the radioactive amino acids had been rapidly incorporated into a small fraction of the plasma proteins in the liver before being absorbed by the mammary gland was excluded since the same results were obtained when an isolated mammary gland was perfused with blood (Barry 1958).

It is difficult to measure changes in the specific activity of an amino acid in wool over periods of only a few hours. Fleischer, Vidaver, and Haurowitz (1959) have measured changes in the optical density along radioautograms of rat hairs labelled with ^{35}S , but have not shown whether the absolute value of the specific activity can be determined accurately by this technique. In the present work it has been possible to show that the cystine in wool attained a much higher activity than the cystine of the plasma proteins when a sheep was given an intravenous dose of DL- ^{35}S cystine. Cystine, therefore, is probably not incorporated in wool by a process involving a direct modification of the plasma proteins. However, the possibility that one or more minor components of the plasma proteins are precursors of wool keratin has not been definitely excluded.

II. MATERIAL AND METHODS

A Corriedale ewe (52 kg body wt.) was injected intravenously with DL- ^{35}S cystine (21.7 mg, 829 μC , obtained from the Radiochemical Centre, Amersham, England) as the hydrochloride in 10 ml 0.9% NaCl. The cystine was shown to be radiochemically pure within experimental error ($\pm 2\%$) by carrier dilution analysis. Samples of blood and wool were taken at intervals in order to compare the maximum activity attained by the cystine in the plasma proteins and the wool protein. To determine the time at which the maxima occurred, the specific activities of the proteins themselves were measured first. The cystine was then isolated from hydrolysates of representative samples of the mixed plasma proteins and of the wool, and its specific activity measured. To build up a more complete picture of the fate of cystine in the sheep, samples of plasma were also analysed for total ^{35}S and for ^{35}S as free cystine and sulphate.

(a) *Measurements of Specific Activity*

Except for the wool, all samples were counted at "infinite" thickness on 1 cm² polythene disks (Popják and Beeckmans 1950) or the results corrected to the infinite thickness value where necessary from an empirically constructed correction curve. A thin end-window G.M. counter (G.E.C. type EHM2S) and an Ekco Scaler (N529) were used, at least 2500 counts being taken for each sample. Resolving time and background corrections were made and the net counting rates were further corrected for decay and for possible sensitivity changes in the counter by counting ^{35}S cystine standards with each batch of unknowns. Material with a specific activity of 1 $\mu\text{C/g}$ gave 730 counts/min.

(b) *Analysis of the Blood Samples*

The blood samples were centrifuged at 2700 *g* for 30 min at 5°C, and the plasma either analysed immediately or frozen until required.

(i) *Total ^{35}S Content.*—Plasma samples (1 ml) were evaporated to dryness under an infrared lamp, ground to a powder, and the specific activity measured.

(ii) *Trichloroacetic acid-precipitable ^{35}S .*—Plasma samples (1 ml) were added to trichloroacetic acid (TCA) (4 ml 10% w/v), allowed to stand at room temperature for 2–3 hr, and the precipitated proteins centrifuged, washed twice with TCA (10 ml 5% w/v), once with ethanol (95%), once with ethanol-ether (1 : 1 v/v), and finally with ether. The dried, finely ground TCA precipitates contained both the [^{35}S]cystine incorporated in the protein chains and that bound to the proteins through disulphide bonds. The amount of bound ^{35}S was found to increase considerably *in vitro* during the first few hours after taking the early blood samples.

(iii) *Protein ^{35}S .*—To remove the bound cystine other samples (1 ml) of the plasma were mixed with aqueous NaHSO_3 (0.2 ml 10%), or with mercaptoethanol (Lee *et al.* 1951) for 5 min at room temperature before the TCA precipitation. The ^{35}S in the product of this treatment was considered to be “protein- ^{35}S ”, and the amount of bound ^{35}S was calculated as the difference between the TCA-precipitable ^{35}S and the protein ^{35}S .

(iv) *^{35}S as Free Cystine.*—The “carrier” technique was used because of the low concentration of cystine in plasma. Samples of plasma (1 ml) were added to TCA (5 ml 10%) containing non-radioactive DL-cystine (40 mg). The mixtures were allowed to stand overnight before isolating the TCA precipitates as described above. The TCA filtrate and washings were extracted twice with ether to remove most of the TCA and the pH adjusted to 5 with dilute NH_4OH to precipitate the cystine. An equal volume of ethanol was added to aid the precipitation and the cystine was filtered, washed, dried, and its specific activity measured. From the specific activity and the known mass of cystine added the amount of ^{35}S in the plasma as free cystine was calculated.

(v) *^{35}S Sulphate.*—The carrier technique was also used. The filtrate and washings from the cystine precipitation (Section II(b) (iv)) were recovered quantitatively and mixed with carrier sulphate ions in the form of aqueous K_2SO_4 (10 ml 0.0236M). Benzidine hydrochloride (4 ml 2%) and ethanol (final concentration 50%) were then added and the precipitate of benzidine sulphate filtered, washed well with aqueous ethanol, dried, and its specific activity measured.

(c) The Wool Samples

(i) *Preparation of the Wool.*—The wool was clipped from the sheep as closely as possible about 18 hr before the injection. Four areas on the skin were then defined by tattooing. Area 1 (18×44 cm) was shaved at intervals after the injection (cf. Fig. 3). Areas 2 (17×29 cm), 3 (13×37 cm), and 4 (26×27 cm) on the other side of the sheep were clipped as closely as possible 1, 2, and 3 days after the injection. Each of these three areas was then clipped every 3 days to give successive 3-day wool samples. Twenty days after the injection the four areas and the rest of the fleece were clipped. Since the clipped wool gave substantially the same results as the shaved wool during the first 20 days, one area only (area 1) was clipped at intervals for a further 70 days.

The wool was isolated from the soapy suspension resulting from the shaving of area 1 by centrifugation at 100 *g* for about 10 min. Most of the supernatant solution was decanted and the residue filtered and washed well with distilled water, ethanol, and ether. The clipped wool from the other areas was transferred directly to a filter paper and washed well with ether, ethanol, and water.

(ii) *Measurement of the Specific Activity of the Wool.*—Infinitely thick samples of the wool, cut into lengths of a few millimetres if necessary, were spread as evenly as possible on 1-in. dia. aluminium planchets, dried in an oven at 110°C, allowed to cool in a desiccator, and counted for no more than 10 min after being removed from the desiccator. This method gave sufficiently accurate results. For example, 10 such samples prepared from the same batch of wool gave the following counting rates: 1180, 1160, 1130, 1130, 1110, 1090, 1150, 1210, 1140, and 1180 counts/min; mean 1150 counts/min with a standard deviation of 32 counts/min. Some of the same wool was oxidized (Walkenstein and Knebel 1957) and the ³⁵S assayed as benzidine sulphate on 1-cm² polythene disks. It was calculated from these results and those from benzidine sulphate prepared by oxidizing samples of [³⁵S]cystine that wool with a specific activity of 1 μ c/g counted directly on the aluminium planchets gave 2300 counts/min.

(g) *Isolation of Cystine from the Wool and Plasma Proteins*

Samples of the plasma proteins and of wool (about 1 g) were hydrolysed by refluxing with HCl (200 ml 6*N*) for 16 hr. The bulk of the HCl was removed by vacuum distillation and the hydrolysate added to a column of "Amberlite IR120" (2.2 by 150 cm) and eluted with HCl (Hirs, Moore, and Stein 1954). The fractions containing cystine were combined, evaporated to dryness *in vacuo*, and dissolved in a few ml water. This solution was filtered and the pH adjusted to 5 with NH₄OH to precipitate cystine, which was filtered, washed with water and ethanol, dried, and its specific activity measured. Paper chromatograms showed that cystine isolated in this way usually contained traces of leucine or isoleucine or both. However, removal of these impurities by reprecipitation of the cystine produced a negligible change in its specific activity.

The results for cystine were confirmed by measuring the specific activity of the cysteic acid isolated from samples of wool which had been oxidized with peracetic acid (Corfield, Robson, and Skinner 1958) or from plasma proteins oxidized with performic acid (Bidmead and Ley 1958). The cysteic acid was isolated by elution from columns of "Amberlite IR120" as described above. Under these conditions cysteic acid was eluted first, free of contamination by any other amino acid. The cysteic acid fraction was evaporated to dryness *in vacuo*, dissolved in water, filtered, and diluted with ethanol. The crystals which separated were filtered, washed with ethanol, dried, and the specific activity measured. Recrystallization from aqueous ethanol did not alter the specific activity.

III. RESULTS

The radiochemical composition of the plasma from the blood samples taken during the first day after the dose of DL-[³⁵S]cystine is shown in Figure 1. Initially

the total and TCA-precipitable radioactivity were measured. However, the specific activities of the TCA precipitates from the first few samples were higher than expected; that is, they did not produce a curve rising from zero to a maximum. Moreover, although stored in the frozen state, each time the samples were thawed and analysed, the specific activities of the first two TCA precipitates (from the blood

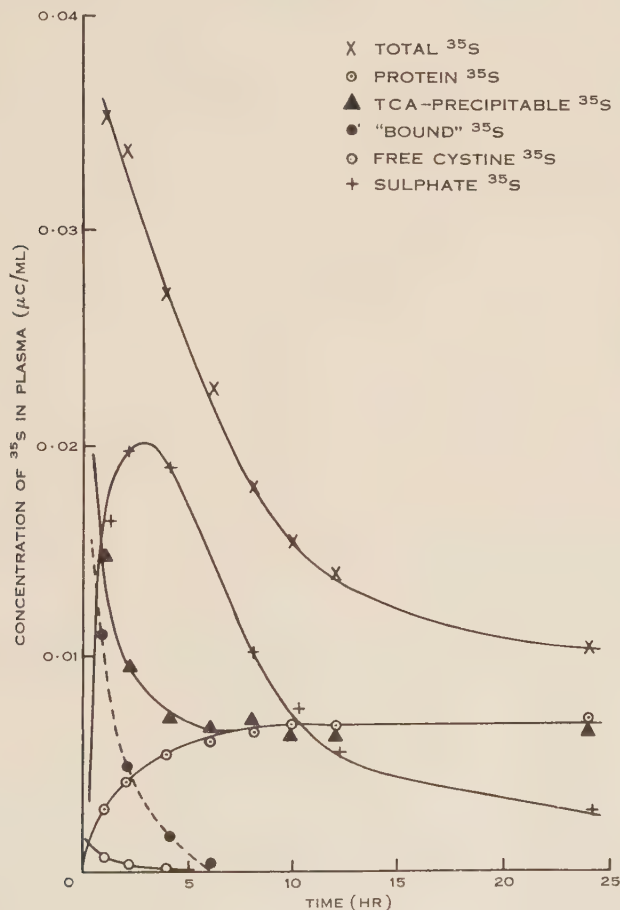


Fig. 1.—Amounts of ^{35}S present in various forms in the plasma from blood samples taken during the first day after the intravenous injection of DL-[^{35}S]cystine into a sheep. The results were obtained at the time of analysis for sulphate ^{35}S by which time the bulk of the non-peptide cystine had become bound to the proteins by disulphide bonds. The amount of bound ^{35}S was calculated by subtracting the protein ^{35}S from the TCA-precipitable ^{35}S .

samples taken 1 and 2 hr after the injection) had increased, whereas the later samples were unchanged. These anomalous results were shown to be due to the binding of cystine to the plasma proteins by disulphide bonds. Separate experiments, in which DL-[^{35}S]cystine was added to plasma *in vitro*, showed that a large proportion of the cystine added to plasma becomes bound and that the bound cystine could be removed with a reducing agent. Eight weeks after the samples were taken the distribution of ^{35}S was as shown in Figure 1.

After about 6 hr the protein- ^{35}S and TCA-precipitable- ^{35}S curves were identical, showing that a negligible amount of the ^{35}S was present as non-protein cystine after that time. A large proportion of the cystine- ^{35}S was converted to sulphate- ^{35}S , the peak concentration of which occurred after about 3 hr. The bulk of the radioactivity (more than 90%) was thus accounted for as protein, sulphate, and in the early stages, free and protein-bound ^{35}S (presumably still cystine).

The specific activity of the mixed plasma proteins reached a maximum value about 10 hr after the injection and then decreased exponentially with a half-time of about 20 days (Fig. 2). This figure also shows that the ratio of the specific activities of the dried plasma and plasma proteins did not become constant for about 3 days. After this time the bulk of the circulating ^{35}S was evidently present in the proteins, most of the sulphate- ^{35}S having been excreted.

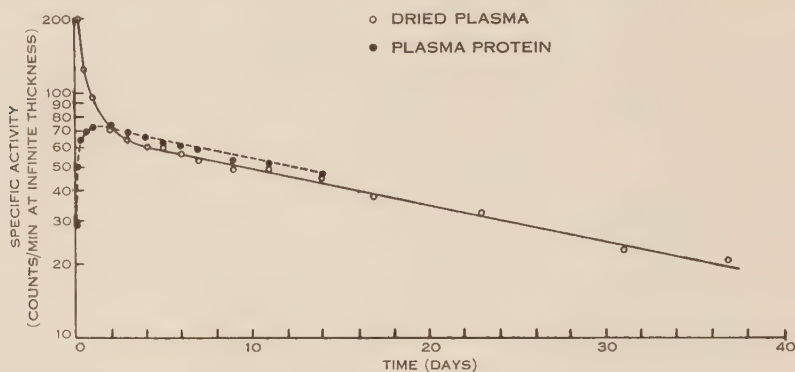


Fig. 2.—Changes in specific activity of the dried plasma and plasma proteins following an intravenous dose of DL- ^{35}S cystine. The slope of the linear part of the curves indicates that the plasma proteins were turned over with a half-time of about 20 days.

The maximum counting rate of the plasma proteins was 73 counts/min, which corresponded to $0.10 \mu\text{c/g}$. Assuming a plasma volume of 3 l. and a protein content of 7%, about 2.5% of the dose was incorporated into the plasma proteins.

The counting rates of the wool samples from area 1 are shown in Figure 3. Radioactivity was first detected in the third day's shavings. A rapid rise to a maximum specific activity on the sixth day was followed at first by a rapid and then a more gradual decline. The declining portion of the specific activity–time curve was resolvable into three exponential curves, as shown in Figure 3, with half-times of about 26, 3, and 1 days respectively.

Substantially the same results were obtained for the wool clipped from the other tattooed areas. Since the average specific activity of the wool grown during the first 20 days after the dose was $0.66 \pm 0.02 \mu\text{c/g}$, and the total weight of wool collected was 119 g, it may be calculated that at least 9.5% of the radioactive dose appeared in the fleece during this period. The true value must be somewhat higher because the wool was not clipped from the head and legs of the animal. A similar calculation showed that the wool grown from the 20th to the 90th day contained a further 1.9% of the dose.

The specific activities of the cystine and cysteic acid isolated from various samples of wool and plasma proteins are shown in Table 1, together with those for benzidine sulphate prepared from completely oxidized wool. To compare the results more easily the specific activity of the *sulphur* in each sample was calculated and is shown in the table. Since the bulk of the sulphur in wool is present as cystine (Simmonds 1954) and since the specific activity of the sulphur in the

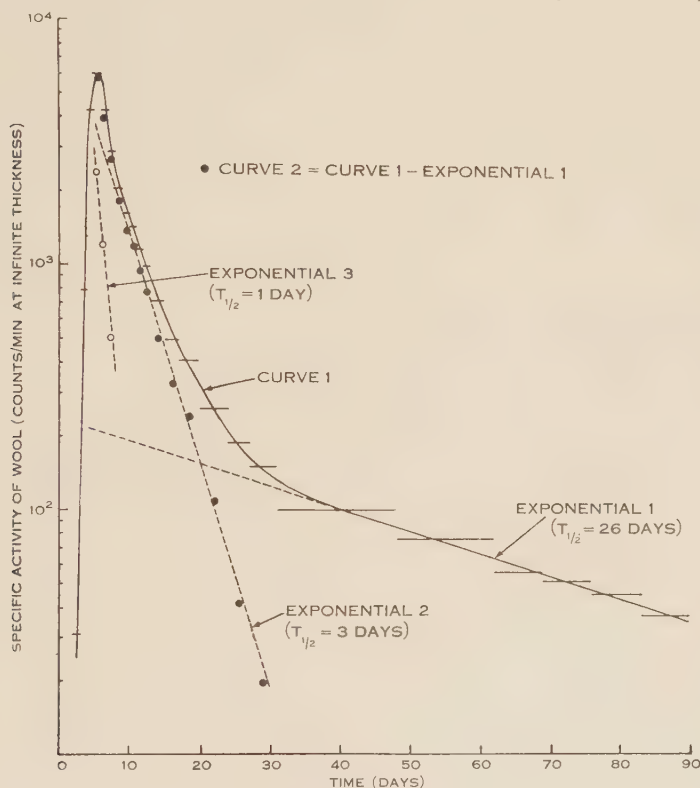


Fig. 3.—Graph showing the specific radioactivity of wool clipped at intervals from the same region of a sheep after an intravenous dose of DL- ^{35}S cystine. The horizontal lines represent the average net counting rates, corrected for decay, of infinitely thick wool samples grown during periods of time indicated by the length of each line. The descending portion of the curve has been resolved into three exponentials, which are discussed in the text.

cystine isolated from the first 20 days wool was slightly higher than that of the total sulphur from the same wool, it follows that substantially all the ^{35}S in this wool was accounted for as cystine. Similarly all the ^{35}S in the plasma proteins examined was evidently present as cystine, since no other ^{35}S was observed in the effluent from the ion-exchange separations of the protein hydrolysates and since a reasonable value (3.3%) for the percentage of cystine in plasma proteins could be calculated from the results on the assumption that all the ^{35}S was present as cystine.

The maximum observed specific activity of the cystine in the wool was about seven times higher than the maximum for the cystine in the plasma proteins. Similar results have been obtained in two other experiments.

IV. DISCUSSION

The results (Table 1) show that the cystine in the wool reached a much higher specific activity than the maximum attained by the cystine in the plasma proteins—about seven times higher. Radioautographic studies of the labelled wool fibres

TABLE 1

SPECIFIC ACTIVITIES OF VARIOUS SAMPLES ISOLATED FROM WOOL AND PLASMA PROTEINS
Individual values given for benzidine sulphate are for separate oxidations; those for cystine and cysteic acid are for different portions of the effluent containing these compounds from each ion-exchange separation

Compound Assayed	Source	Specific Activity ($\mu\text{c/g}$) of:	
		Compound Assayed	Sulphur in Compound Assayed (calc.)
Cystine	Material injected	38,200	143,000
Benzidine sulphate	First 20 days' wool	1.96 } 2.11 } 2.03	17.9
Benzidine sulphate	First 20 days' wool oxidized with peracetic acid	2.02 } 2.07 } 2.05	18.1
Cystine	First 20 days' wool	5.07 } 4.75 } 5.03 } 5.05 5.36 }	19.0
Cysteic acid	First 20 days' wool oxidized with peracetic acid	3.56 } 3.88 } 3.72	19.6
Cystine	Wool grown during the 5th, 6th, and 7th day	18.2 } 18.5 } 18.3	68.5
Cystine	Plasma proteins isolated from blood collected 6 days after dose	2.83 } 2.87 } 3.02 } 2.91	10.9
Cysteic acid	Plasma proteins isolated from blood collected 4 days after dose, then oxidized with per-formic acid	1.94 } 1.98 } 1.96	10.3

have shown that the region of maximum specific activity corresponds to less than 1 day's wool growth (Downes and Lyne 1959). Therefore, the true maximum specific activity of the wool cystine must have been much higher than the observed value because the latter was the average for a 24-hr period, not including all of the most radioactive segments of the fibres.

This comparison suggests that a large proportion of the cystine in wool keratin is incorporated, not by any process involving a modification of plasma proteins, but probably directly as the free amino acid. The alternative possibility is that the labelled cystine was concentrated in a component of the plasma proteins with a higher specific activity than was observed in the cystine of the wool. Although insufficient plasma was available to examine this point, fractionations of plasma proteins similarly labelled with [^{35}S]cystine in subsequent experiments have since been carried out as described by Campbell and Work (1952). Radioactivity was present in the fibrin, globulin, and albumin fractions of the plasma obtained 6 and 30 days after an intravenous dose, and their specific activities did not differ by more than a factor of about 2 (Downes, unpublished data). All the radioactivity is therefore not concentrated into a minor component of the plasma proteins. A more detailed study of the labelling patterns of the various plasma proteins is now being made.

Tarver (1954) has pointed out that the non-peptide binding of radioactive amino acids by proteins can be especially troublesome with labelled cystine, and other authors have referred specifically to the binding of cysteine by plasma proteins (Lee *et al.* 1951; Samarina, Kritzman, and Konikova 1956). Cysteine can evidently be bound as half-cystine residues by disulphide bonds and removed by reducing agents such as mercaptoethanol. However, as may be seen from Figure 1, there was a negligible amount of radioactive free or bound cystine in the plasma from about 6 hr after the injection. After this time, pretreatment with a reducing agent did not alter the specific activity. The plasma protein samples used to measure the specific activity of the incorporated cystine were prepared from blood taken from the sheep at least 4 days after the dose, by which time practically all the circulating ^{35}S must have been present as cystine incorporated into protein chains by peptide bonds. This is evident from Figure 2 which shows that the ratio of the specific activity of the plasma proteins to that of the dried plasma was constant from approximately 3 days after the injection. Such proteins must have contained some bound cystine but the error introduced would have been small since other work (Downes, unpublished data) has shown the mass of the bound cystine to be less than 5% of that of the peptide-linked cystine.

There is therefore little doubt that the [^{35}S]cystine was incorporated in peptide linkage in the plasma proteins referred to in Table 1. Similarly, all the labelled cystine found in the wool keratin must have been incorporated in the protein chains otherwise the oxidation with peracetic acid would have removed some of the radioactivity.

There are two observations which suggest that at least some of the cystine in wool is incorporated during the final keratinization and the present results support this hypothesis. Firstly, radioautographic studies in the lamb (Ryder 1959) and mouse (Harkness and Bern 1957) showed an intense concentration of radioactivity in the keratogenous zone soon after the administration of an intraperitoneal dose of labelled cystine; and secondly, cystine occurs in hair roots in much lower concentrations than in fully keratinized hair (Rogers and Simmonds 1958). In view of the exceptional role played by cystine in keratinization, the conclusion that the

cystine in wool keratin is largely derived from the free amino acid may not be applicable to the other amino acids.

Finally, what is the significance of the three exponential curves shown in Figure 3? The graphical analysis of a specific activity curve in the way shown may be taken as an indication of the number of metabolic "pools" with which the substance being studied equilibrates (see, for example, Matthews 1957 for the application of this method to labelled plasma proteins). Since wool is an excretion product which is formed at a steady rate we may presume that changes in its specific activity reflect changes in the specific activity of the material from which it is synthesized. A straight line could reasonably be drawn through the points representing the mean specific activities of the wool samples grown from the 35th to the 90th day. This line, extrapolated back to zero time on the graph presumably indicates the results that would have been obtained if the injected cystine had been mixed instantaneously with all the other cystine in the animal, excluding that in the wool already grown. Evidently equilibrium was not reached until about the 35th day, after which time the specific activity fell exponentially with a half-time of about 26 days. This half-time, which is of the same order as that for the plasma proteins, probably indicates the average turnover half-time of cystine in the sheep.

If free cystine in the plasma were the only direct precursor of the cystine in wool and if it had been possible to measure the instantaneous specific activity of the cystine in wool continuously, then one would have expected to find a high specific activity in the cystine incorporated in the wool immediately after the injection followed at first by a rapid fall during the first day or so and subsequently by the slow exponential disappearance with a half-time of 26 days. In practice, with daily shavings as a poor substitute for instantaneous measurements, the first part of such a curve would have been masked by the delay of a few days taken for the radioactive portions to emerge above the skin and by the fact that these portions do not all emerge at the same time. Nevertheless, in these circumstances one would have expected the descending portion of the specific activity curve to be resolvable into two exponentials only—a rapid drop which would be a reflection of the ascending portion of the curve and would therefore have indicated an apparent half-time of only a day or so; and the slow component representing the overall turnover of cystine in the whole animal.

The presence of the line with an intermediate half-time of about 3 days suggests that there is an intermediate metabolic pool between the free cystine in plasma and the cystine in wool. As pointed out above, a half-time of 3 days seems much too long to be associated with the free cystine in the plasma. Free amino acids in plasma are known to have turnover half-times of only a few minutes and unpublished experiments carried out here have shown that the exchange between free and bound cysteine in plasma occurs with a half-time of about 1 hr. This is confirmed by Figure 1 which shows that there was a very small amount of ^{35}S circulating as free or bound cystine about 6 hr after the dose. Provided there were no large differences in the maximum specific activities of the cystine in the individual plasma proteins, the specific activity of the free plus bound cystine would have been less than the maximum specific activity of the cystine in the plasma proteins,

after this maximum was attained. This follows from the relationship between the specific activity of a product and its precursors (Zilversmit, Entenman, and Fishler 1943). The maximum specific activity of the cystine in the plasma proteins was reached in 24 hr at the latest and must have been about 3 $\mu\text{c/g}$ (Table 1), but the specific activity of the cystine in the wool did not fall to this value until about the 15th day after the injection. Allowing for the average delay of about 6 days taken for the radioactivity to appear above the skin surface it may be concluded that the specific activity of the wool cystine was higher than that of the free cystine of the plasma during the first 9 days.

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STUDIES ON THE RATE OF WOOL GROWTH USING [³⁵S]CYSTINE

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[Manuscript received July 28, 1960]

Summary

The radioautographic method has been used to study the length growth rate of wool fibres in a sheep subjected to changing nutritional conditions.

The reproducibility of radioautographic length measurements by different observers has been examined in a detailed study of 10 fibres. The variance between observers was negligible compared with the variance between fibres.

A Corriedale wether was given nine intravenous doses of DL-[³⁵S]cystine at 4-day intervals. When the food intake was increased from 800 g to about 1300 g per day the absolute increase in length growth rate was similar for all fibres. When the level of nutrition was reduced to 300 g per day the length growth rate decreased in proportion to the initial rate. Although the changes in volume were approximately proportional to the initial growth rate, a differential rate of response in the largest and smallest fibres was observed. Thus the length and diameter components of volume responded independently to the nutritional changes. Clipping alone had little or no effect on the rate of wool growth when changes in skin temperature were reduced to a minimum.

Experience has shown that small doses of radioactivity can be used in studies of this type. The sensitivity of the radioautographic technique is discussed.

I. INTRODUCTION

The advantages of the radioautographic technique for studying the rate of wool growth were indicated in a study by Downes and Lyne (1959) in which intravenous injections of ³⁵S-labelled cystine were used to label the whole fleece. It was shown that the growth in length of individual wool fibres could be measured with good accuracy over short periods, of the order of a few days, without having to remove the fibres from the sheep until the end of the experiment.

In the present work the radioautographic technique was used to determine the effect on wool growth of changes in food intake and of clipping. At the same time, the accuracy of the method was examined more critically.

II. MATERIAL AND METHODS

(a) *Experimental Procedure*

A Corriedale wether (40 kg body weight) was kept in a metabolism cage in a room at $23 \pm 1^\circ\text{C}$ to eliminate the influence of air temperature on wool growth. The animal was initially covered uniformly by an 8-cm thick fleece. The left side was clipped in the 2 hr before the first injection. Three small areas (A, B, and C, each about 5 by 5 cm) and two larger areas (I and II, each about 10 by 10 cm) of mid-lateral abdominal skin were clipped closely with care and defined by tattooing;

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their anteroposterior order was A, I, B, II, C. About 3 hr after the first injection a sheepskin, with its 3-cm thick fleece inwards, was used to cover the left side of the sheep. The skin was kept on for the duration of the experiment, except during the clipping, in order to minimize possible changes in the rate of wool growth due to changes in skin temperature, and thus to enable any other effects of clipping to be studied. Skin temperatures (measured with copper-constantan thermocouples held against the skin with an adhesive) on both sides of the animal were identical ($38.3 \pm 0.3^\circ\text{C}$) within 3 hr.

Nine doses of DL- ^{35}S cystine were injected at 4-day (± 15 min) intervals into the left jugular vein and washed in with 10 ml 0.9% NaCl. The cystine (30.9 mg, 19.5 mc/m-mole at the time of the first injection) was dissolved in the minimum

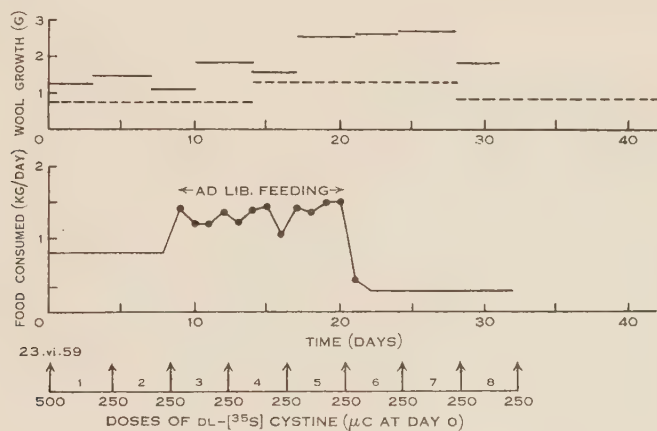


Fig. 1.—Feeding regime, timing and amount of each radioactive dose, and wool clipped. Periods between successive doses are numbered 1–8 as shown. — — — Mass of clean dry wool clipped from areas I and II (g/14 days); ——— masses (g/day) clipped from the rest of the left side excluding the tattooed areas.

amount of HCl and diluted with 0.9% NaCl to 50 ml. Eight 5-ml portions of the stock solution were dispensed into glass tubes and kept frozen until required. The remaining 10 ml was used for the first dose. The radiochemical purity of the cystine (obtained from the Radiochemical Centre, England) was checked at intervals by eluting samples with HCl from a small "Amberlite IR120" column. In every case a single peak corresponding to cystine and accounting for more than 98% of the radioactivity was obtained. Analysis by the carrier dilution procedure confirmed the chromatographic results.

The sheep was eating 800 g per day of equal quantities of lucerne chaff and wheaten chaff for 4 months before the experiment and until the day of the third injection of cystine. From then until the day of the sixth injection, i.e. for 12 days, it was allowed to eat the same ration *ad lib*. On the day of the sixth dose the animal received 450 g, and on each succeeding day until the end of the experiment 300 g of the same ration. The timing of the injections in relation to the food eaten is given in Figure 1.

The wool on areas I and II was clipped fortnightly; it was washed successively with ether, ethanol, and cold water, dried at 100°C, and weighed. Ten days after the last injection wool was clipped from areas A, B, and C, and from three corresponding areas D, E, and F on the right side. Apart from the tattooed areas, the entire left side was clipped every three or four days (Fig. 1) to see if repeated clipping of the surrounding wool produced any indirect effect on the rate of growth of the fibres in the small areas A, B, and C.

(b) *Radioautographic Technique*

Wool fibres from areas A–F were cleaned in ether and dried on filter paper. They were mounted on microscope slides as follows: about 30 fibres were thoroughly wetted in a drop of freshly prepared solution of egg albumin in water (*c.* 0.1 ml,

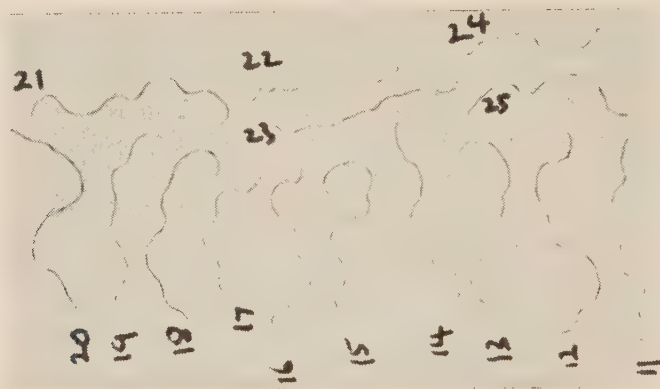


Fig. 2.—Typical preparation for length measurements of radioactive wool fibres. $\times 2.3$.

5% w/v); the fibres were separated in the solution which was smeared over three-quarters of the slide. The slide was baked dry 25 cm below an infrared lamp for 2 hr to ensure that the fibres were held firmly. Each slide was then covered with a radioautographic stripping film ("Kodak AR50") using the technique described by Pelc (1956). After a suitable exposure time the films, still in contact with the fibres, were developed, fixed, washed, dried, and each fibre numbered to enable repeat measurements to be made. Figure 2 shows part of a typical preparation.

The lengths of 100 fibres grown on each area during each of the eight 4-day periods was measured with a flexible rule or a graduated knurled wheel after projection and magnification to $\times 215$ (Downes and Lyne 1959). Most of the fibres measured were radioautographed for 11 days, starting 44 days after they were clipped.

During the length measurements an estimate was made of the amount of medullation along each fibre over each 4-day period. Later, the diameters of the 100 fibres from area B were measured (magnification $\times 500$) at positions corresponding to just before and after each dose. The mean of the two measurements was taken as the diameter of the fibre at the time of each injection. The volume of fibre grown during each period was calculated by assuming that each segment was a cylinder

with a diameter equal to the mean of the diameters at the two ends. The volumes were less accurate than the lengths because the fibres were not perfect cylinders, the diameters could not be measured very accurately, and the amount of medullation increased and decreased after the food intake was respectively raised and lowered.

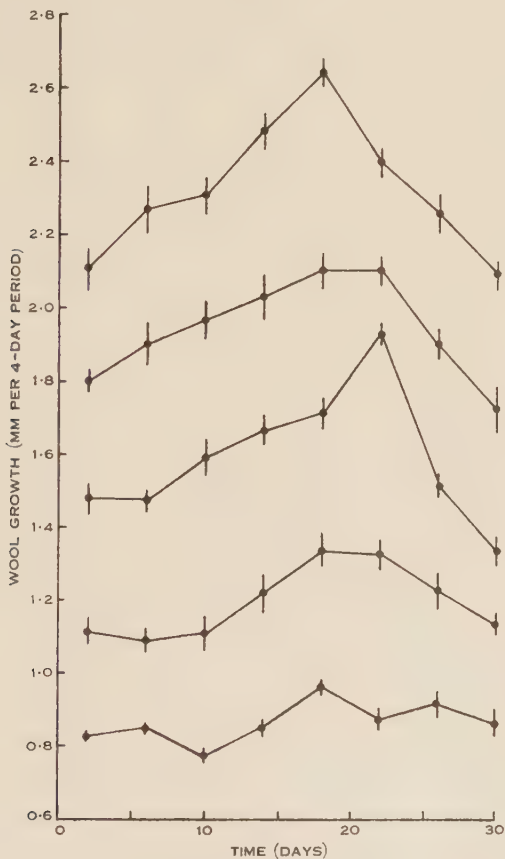


Fig. 3.—Accuracy of radioautographic length measurements of five individual fibres. Each point represents the mean of 10 measurements of length of wool grown during each 4-day period. Standard deviations of single measurements are shown as vertical lines.

III. RESULTS

(a) Accuracy of Method

The lengths of 10 fibres taken randomly from the 100 fibres from position C were measured by five observers on two occasions. Figure 3 shows typical results from five fibres. Most standard deviations of single measurements were less than $\pm 4\%$ of the mean. It is thought that the absolute errors of measurement in the bigger fibres were larger because of the greater difficulty in defining the start of the radioactive band. The mean growth rates for each group of 10 fibres during

each of the eight periods are shown in Table 1. The largest standard deviation from the mean of each set of results was $\pm 1.5\%$. The maximum observer difference, as judged by the mean lengths of the 10 fibres over the whole eight periods, was about 1%.

Nine of the fibres used for the accuracy test were removed from the slides and film, soaked in distilled water for about 15 min, and radioautographed again. The second exposure began 146 days after the commencement of the first, that is 232 days

TABLE 1
REPRODUCIBILITY OF RADIOAUTOGRAPHIC LENGTH MEASUREMENTS BY FIVE OBSERVERS

Period	Mean Length (mm) of Wool Grown by 10 Fibres during the Eight 4-day Periods of the Experiment*										Mean \pm S.D.
	Observer										
	A		B		C		D		E		
1	1.30	1.29	1.30	1.32	1.28	1.27	1.30	1.30	1.30	1.31	1.30 \pm 0.015
2	1.33	1.32	1.36	1.35	1.34	1.38	1.36	1.36	1.32	1.33	1.34 \pm 0.020
3	1.40	1.39	1.40	1.41	1.37	1.38	1.40	1.39	1.39	1.40	1.39 \pm 0.012
4	1.49	1.46	1.47	1.48	1.44	1.48	1.48	1.49	1.49	1.43	1.47 \pm 0.022
5	1.58	1.57	1.56	1.57	1.59	1.57	1.58	1.59	1.54	1.59	1.57 \pm 0.016
6	1.51	1.52	1.55	1.54	1.51	1.53	1.56	1.52	1.54	1.55	1.53 \pm 0.018
7	1.43	1.43	1.45	1.46	1.46	1.46	1.46	1.48	1.42	1.45	1.45 \pm 0.018
8	1.33	1.35	1.34	1.32	1.32	1.29	1.33	1.33	1.33	1.31	1.32 \pm 0.018
Total length	11.37	11.33	11.43	11.45	11.31	11.36	11.47	11.46	11.33	11.37	

* Each observer measured the same 10 fibres, selected at random from position C, on two different occasions.

after the first dose, and was increased to 41 days because of the decay of the ^{35}S in the intervening period. The length measurements were then repeated by two of the observers and were found to be the same as the original ones within experimental error.

(b) Wool Growth and Nutrition

The mass of wool grown on all areas was related to the amount of food eaten (Fig. 1). This is best seen from the masses obtained from areas I and II. The masses obtained from the rest of the left side of the sheep were much less reliable because of the difficulties in clipping wool reproducibly and quantitatively at intervals of only 3 or 4 days. In view of the inaccuracy of the results from these clippings, it is considered that the apparent lag which they indicate in the response to the reduced plane of nutrition is not significant.

The results of the radioautographic length measurements and especially the rapid response of wool growth to changes in the plane of nutrition are shown in Figure 4. The average length of wool grown by 600 fibres, 100 from each of the six

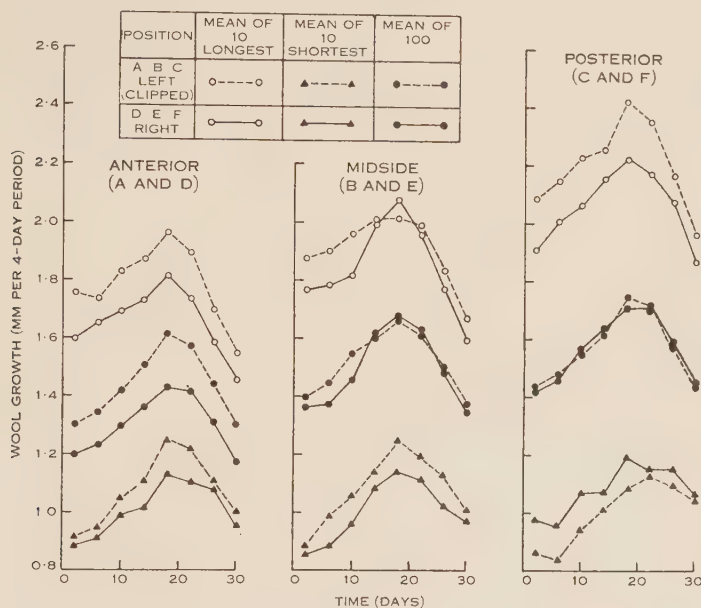


Fig. 4.—Results of the radioautographic length measurements on 100 fibres from each of the six positions (A-F) during the eight 4-day periods.

areas (A-F), was 1.35 (S.D. ± 0.31) mm during the first 4 days. Maximum growth of 1.64 (± 0.34) mm occurred during the last 4 days of the *ad lib.* feeding, representing

TABLE 2
MEAN LENGTHS AND STANDARD DEVIATIONS OF 100 FIBRES FROM
POSITIONS A-F DURING PERIODS 1 AND 8

Position		Mean Length (mm) \pm S.D.	
		Period 1	Period 8
Anterior	A	1.30 ± 0.26	1.30 ± 0.18
	D	1.19 ± 0.24	1.17 ± 0.22
Midside	B	1.40 ± 0.33	1.37 ± 0.21
	E	1.36 ± 0.29	1.34 ± 0.22
Posterior	C	1.44 ± 0.39	1.44 ± 0.30
	F	1.42 ± 0.30	1.45 ± 0.25

an increase of approximately 20%. The average growth rate then fell to 1.34 (± 0.25) mm during the last experimental period.

Figure 4 also shows that the changes in nutrition produced different growth patterns in fibres of different length, namely the rate of growth of the 10 longest fibres from all areas during the last period was below that of the first period, whereas the 10 shortest ones were still growing at rates above the initial rate. This was confirmed by calculating the standard deviations of the lengths for each group of 100 fibres during periods 1 and 8. In every case the standard deviation was larger

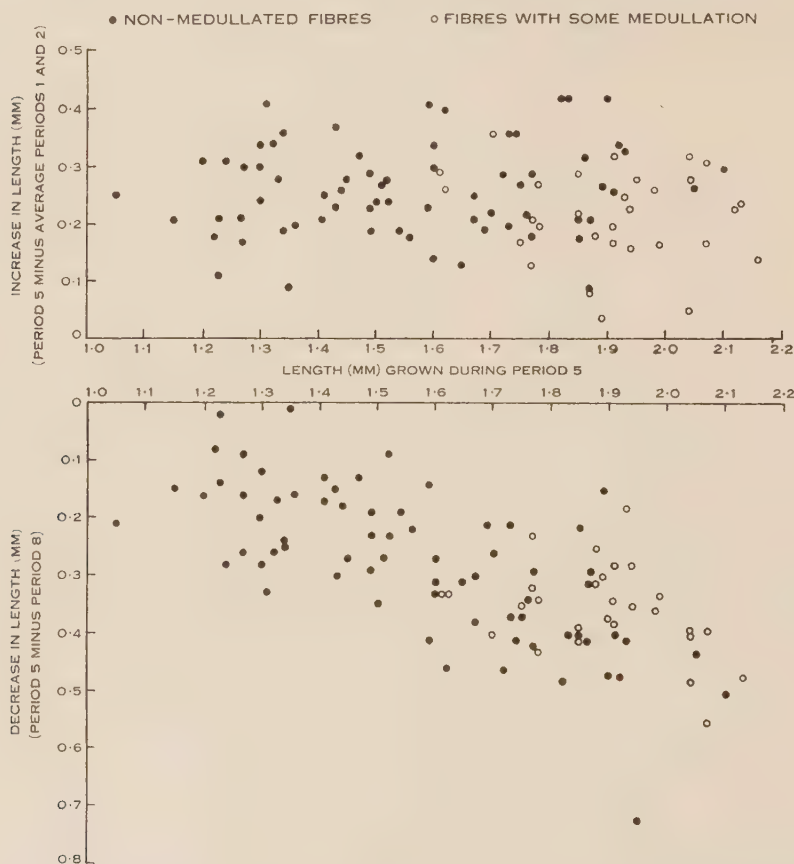


Fig. 5.—Changes in length of the 100 fibres from position B. Each point in the upper figure shows the difference between the length grown during period 5 and the average length for periods 1 and 2. The lower figure shows the corresponding differences for periods 5 and 8.

for period 1 than for period 8 (Table 2). Figure 5 illustrates these differences in another way; the average increase in *length* of wool grown as a result of the increased plane of nutrition was about the same for all fibres, irrespective of the presence or absence of medullation; that is the percentage increase in length was greater for the smaller fibres. The response to the sudden drop in nutrition was different: the bigger the fibre the larger was the decrease in length and this change was approximately proportional to the fibre length. Similar results were obtained for the other areas.

Figure 6 shows a comparison of the length and volume growth rates for the 100 fibres from area B. Subject to the errors described in the methods, the distribution of values for both length and volume were markedly less during the last period compared with the first.

The true volumes of keratin produced by the medullated fibres must be less than the calculated values, because the medulla contains air spaces (Wildman 1954). Only fibres whose initial growth rates were higher than $8 \times 10^{-4} \text{ mm}^3$ per 4-day period were medullated, but no corrections for the medullation changes were made.

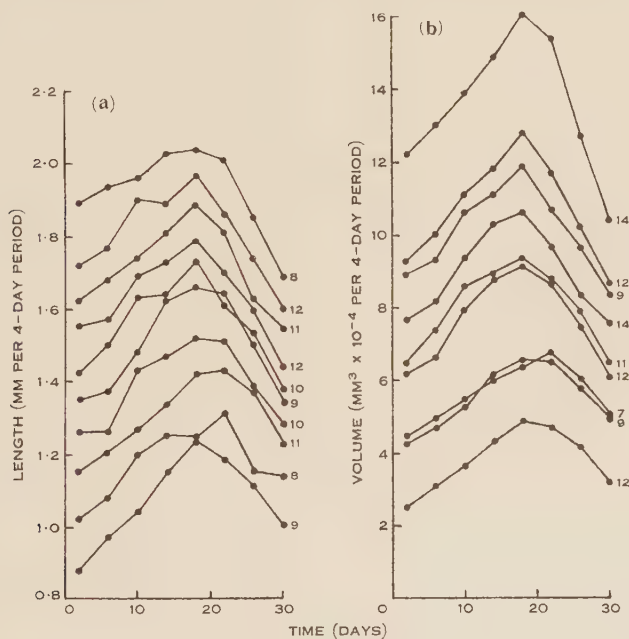


Fig. 6.—Comparing the changes in length and volume for the 100 fibres from position B. (a) Fibres were classified into 10 length groups according to the average length grown during the first two periods (<1.0 , $1.0-1.1$, $1.1-1.2$, etc. and >1.8 mm per 4-days), and the mean lengths for each group during each period calculated. (b) Fibres were classified into nine diameter groups according to the average diameter during the first two periods (<20 , 21 and 22, 23, 24, 25, 26, 27, 28 and 29, and 30–32 μ), and the mean volumes calculated. A similar set of volume curves was obtained when the fibres were classified by lengths as in (a). The number of fibres in each group is shown at the right of each diagram.

Nevertheless, the results showed that the increase in the volume growth rate after the increased food intake was not directly proportional to the initial rate. The average increase was 61% for the smallest 10 fibres and 43% (an upper limit because of the changes in medullation) for the largest 10 fibres from each of the six areas.

(c) *Effect of Clipping*

During the period between the first and second injections, the only period when the effect of clipping could be measured directly, the mean lengths of 300 fibres

from each side of the sheep differed by 4% but this difference was not statistically significant.

Clipping the left side of the sheep every 3 or 4 days did not influence the rate of growth of the fibres clipped from the small areas A, B, and C 10 days after the last injection, when compared with fibres from corresponding positions on the other side of the animal. It is concluded that the direct or indirect effect of clipping, if any, is very small.

IV. DISCUSSION

The results reported here and in our preceding paper (Downes and Lyne 1959) show that the radioautographic technique can be used to measure changes in wool growth rates over short periods with good accuracy. The main source of error is the difficulty of locating the points on each fibre corresponding to the injection times. Experience has shown that this can be done to within $\pm 30 \mu$, which corresponds to about ± 2 hr for fibres growing 0.36 mm per day. Since this is approximately a constant error, the longer the interval between doses the greater is the accuracy of the length measurement. The length of a single fibre grown over a 4-day period can be measured with a standard error of less than $\pm 2\%$ by taking the mean of several measurements as indicated above. For an 8-day period the error would be $\pm 1\%$. However, in studies of a fibre population, a larger number of fibres may be measured with a lower accuracy since the variance between observers is negligible compared with the variance between fibres.

In using the radioautographic technique, the number of fibres that need to be measured will depend on the purpose of the experiment. If an accurate value for the mean length growth rate of the whole fibre population is required then, as with conventional techniques, several hundred fibres must be measured. However, one of the main advantages of the technique is that it enables relative measurements to be made over successive short periods, of the order of 3 or 4 days. In this case a few fibres are sufficient. The results confirm this since the percentage changes in length were practically the same for 10 fibres taken at random as for the whole hundred in each group, even though the individual fibres did not respond in exactly the same way.

The experience gained has shown that much smaller doses of radioactive cystine could have been used. For example, fibres exposed to the radioautographic film about three half-lives after the first injection still produced sufficiently clear "spots" with exposures of about 40 days. Thus the standard dose used, 250 μc of DL-cystine, could have been reduced to about 30 μc provided the fibres had been radioautographed without delay. In addition, since the sheep evidently does not use D-cystine (Downes, unpublished data), the dose could presumably have been halved again if the pure L-isomer had been injected.

Rougeot (1959) has shown that it is possible to label the wool fibres over a small area of skin by a subcutaneous dose of much smaller amounts of [^{35}S]cystine. In his experiment doses of 1–10 μc were injected at 21-day intervals, without producing any visible lesions. However, to our knowledge, it has not been shown whether subcutaneous injections can be made into the same region of the skin at intervals of only a few days without altering the rate of wool growth in that area. Since some

local damage must be produced and since cystine is known to concentrate in healing tissue (Williamson 1959) it is possible that the rate of wool growth could be affected. Intravenous doses, on the other hand, although requiring more radioactivity, label the whole fleece and can be given at convenient sites well away from the regions to be studied.

The biggest fibres grew about three times as fast as the smallest. To explain this, a mechanism for wool growth could be postulated in which the growth stops or becomes very slow for periods of time which might differ for the various follicles. If the extraction of cystine from the blood by the follicles also stops or becomes negligible during such periods, and if these periods are distributed randomly in time one would expect to find fibres with one or more of the radioactive areas missing. However, since every fibre examined showed the nine radioactive spots, and since the time interval between an injection of labelled cystine and the first arrival of the radioactivity at the site of incorporation is no more than a few minutes (Downes and Lyne 1959) the fibres were evidently all growing at the time of each injection. Moreover, in spite of the individual variations, the fibres all responded qualitatively in the same way to the nutritional changes, so that any irregularities in the growth rate must have been for periods much smaller than 4 days. Since each dose was given at the same time of day (± 15 min) the evidence does not exclude the possibility of diurnal or more frequent variations in the rate of fibre growth.

Figure 4 shows that the response to both an increase and decrease in nutrition was quite rapid—certainly much less than 4 days. Similar changes were observed in the wool taken from all six sites examined. The drop in food intake after the sixth dose was much larger than the increase after the third dose but the average length of wool grown during the last period had only fallen to a value about the same as those for the first two periods. This suggests that the wool growth rate responded more slowly to the decrease than to the increase in the food eaten.

The results in Figures 4, 5, and 6 appear to provide the first direct demonstration of a differential response of length growth rate to changes in nutrition. The absolute increases in length growth rate as a result of the increased nutrition were about the same for all fibres; that is, the shorter the fibre the larger the *percentage* increase in length grown per day. When the nutritional level was reduced the decrease in length growth rate was approximately proportional to the original rate. Although the measurements of volume were much less accurate than those of length, the results showed that the changes in volume were not simply proportional to the initial fibre size; the bigger the fibre the smaller the percentage increase. Moreover, at the end of the experiment there was a smaller distribution of fibre volumes than at the beginning (Fig. 6). Thus there was a differential response of volume as well as of length. These differential responses might have been due to different *rates* of change for the various fibres. If the animal had been allowed to come to a new equilibrium the original relationship between the fibres might have been restored. An experiment to examine this is in progress. The present results suggest that, although nutritional changes may produce minor variations in the *relative* growth rates of different follicles, these growth rates are largely determined by properties inherent in the follicles themselves.

On the basis of indirect evidence, other authors have reported differential responses in diameter. For example, Marston (1955) reported a relatively greater response in the diameters of the "stronger" fibres to a higher plane of nutrition. He also stated that "the length of the fibres is similarly influenced by the rate of wool growth though the extent of the change may vary independently of the mean fibre diameter". Our results support this statement. Short, Fraser, and Carter (1958), as a result of a statistical analysis of the diameters of primary and secondary fibres as measured in skin sections taken from a number of sheep, concluded that primary and secondary fibres did show a differential response. However, their results refer only to changes in diameter.

The results show that the effect of clipping alone, if any, is very small. Since the clipped side was kept covered with a thick fleece any effects of clipping due to changes in skin temperature were probably avoided. The fibres from positions A, B, and C were clipped twice—just before the first injection and 10 days after the last—and could therefore have been affected directly by the clipping only once. The repeated clipping of the surrounding wool on the rest of the left side could have produced indirect effects. However, the difference of 4% between the mean lengths grown in the first period by the 300 fibres taken from each side of the sheep was not statistically significant. The mean difference for the eight periods was also about 4%.

V. ACKNOWLEDGMENTS

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STUDIES ON EXPERIMENTAL DERMAL CYSTS IN SHEEP

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Summary

The growth of seven experimental dermal cysts in four sheep has been studied for periods of up to 83 weeks. The similar growth behaviour of all cysts indicates the reliability of the technique for producing standard epithelium-lined cysts. This has been emphasized by producing the cysts in animals of different genotype.

Growth in surface area of the cysts is accompanied by a decrease in the number of wool follicles per unit area. Histological evidence and the constancy of the secondary/primary follicle ratio indicate that the decrease in density per unit area is the result of separation of follicle groups caused by the growth of interstitial connective tissue.

The lack of evidence of abnormality in the structure and metabolism of cyst skin suggests that the cyst environment has little effect upon the metabolism of the implanted skin. There is no evidence that pressure causes any detectable change in the cyst wall.

The value of epithelium-lined cysts as a biological system which can be used to study growth and metabolism is discussed.

I. INTRODUCTION

Epithelium-lined cysts are not uncommon pathological structures in man. Their aetiology and location vary considerably; for example, they may occur as developmental cysts at lines of embryonic closure, as implantation cysts at sites of mechanical injury, as sebaceous cysts arising from infection and blockage of ducts, and as ovarian cysts. They are common in the jaws, where they may be associated with neoplastic, developmental, and inflammatory lesions.

It is generally thought that epithelium-lined cysts tend to increase in size and that this growth is related to a positive intracystic pressure. This assumed relation has been the basis for several studies on the mechanism of cyst growth. For example, James (1926) and Toller (1948) measured the hydrostatic pressure in cysts of the jaws of humans by means of water manometers and found that the pressures were higher than capillary blood pressure. However, although high hydrostatic pressures have been recorded (Toller (1948) obtained an average value of 70 cm water from 51 cases of dental cysts, range 56·6–95·0 cm water), there is little histological evidence that pressures of such dimensions affect the cyst wall. Stokke (1956) discussed this question and suggested that the recording methods used by Toller and James were responsible for the high intracystic pressures.

The necessity for surgical removal of cysts in man generally prevents any extended studies aimed at relating intracystic pressures to cyst growth and cyst

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wall structure and therefore, in order that the behaviour of cysts could be studied over an extended period, dermal cysts were produced experimentally in sheep. In this study sheep were preferred as experimental animals to rodents, the usual laboratory animals, because:

- (1) The size of sheep permitted a number of cysts to be implanted on each animal in accessible locations.
- (2) Most wool follicles have a long growth phase and can be used as markers to indicate the state of the cyst environment as compared with that of normal skin. The extensive knowledge of wool follicles available (Fraser and Short 1960) is an additional advantage.
- (3) Previous studies (Molyneux 1960) showed that the thickness of sheepskin, compared with that of rat and rabbit skin, facilitated the formation of cysts and that the presence of sweat glands in sheepskin contributed towards a fluid cyst content suitable for pressure recordings.

This paper reports observations made on sheepskin growing for up to 83 weeks as a cyst. The investigation was designed primarily to provide basic information on the behaviour and growth of artificially induced epithelium-lined cysts. It was thought that positive intracystic hydrostatic pressure might influence the structure and density of the wool follicles and other epidermal derivatives in the cyst wall and that this effect could readily be studied by comparing the histology of the implanted skin with that of surface skin. The basic premise of this approach is that the mechanism of growth of epithelium-lined cysts is similar regardless of location and that the results of studies of experimental dermal cysts would be of value in relation to pathological cysts.

II. MATERIALS AND METHODS

Seven epithelium-lined cysts were experimentally implanted in four adult sheep. The animals were not selected with a view to homogeneity, but rather to provide diverse characteristics so that the efficiency of the implantation method could be assessed in various environments. The animals chosen were a Corriedale and three Merinos. Of the latter one had a high and one had a low follicle density while the third was a mutant without sweat glands (Short, personal communication). One of the reasons why sheep were selected as experimental animals was the presence of sweat glands which by secreting into the cyst contributed to the cyst contents and intracystic pressure. To examine the effect of lack of sweat secretion on cyst growth, a Merino without sweat glands was used.

The cysts were implanted on the midside region of the animals under local anaesthesia. The surgical technique developed for producing the cysts and the sampling method have been described (Molyneux 1960). At intervals after implantation the skin covering and surrounding the cysts was closely clipped and the size of the then-prominent cysts measured with calipers. When implanted the cysts were semicircular sacs (formed from a circular piece of skin 4 cm in diameter) but as growth occurred they tended to become spherical (Plate 1, Figs. 1 and 2). The size of the cyst, which included a double thickness of surface skin, was measured

across two normal diameters, the mean value being used to calculate the approximate surface area of the cyst, assuming it to be a sphere.

The cysts were then exposed by an incision through the surface skin and the fluid contents were aspirated into a sterile syringe. Skin samples of cyst walls and surface (control) skin were then obtained with a trephine similar to that described by Carter and Clarke (1957). Samples of intracystic wool were also collected. Biopsies of the cysts were made at approximately 5-week intervals after implantation. Multiple cysts were implanted in two animals and the growth of two cysts was observed for up to 83 weeks.

Examination of Skin Samples

The skin samples were fixed in 5% formol saline and, after paraffin embedding, serial sections (8μ) were cut parallel to the skin surface. The cyst samples were sectioned entirely but most of the control samples were sectioned only to the mid-sebaceous gland level. Some entire sections of each cyst were also prepared (Plate 1). Most sections were stained with haemalum, eosin, and picric acid. To study the distribution of sulphhydryl groups, selected samples were fixed in 1% trichloroacetic acid and stained by the dihydroxydinaphthylsulphide (DDD) procedure of Barrnett and Seligman (1952). Sulphhydryl and disulphide groups together were indicated by reduction of disulphide bonds with sodium thioglycollate at pH 8 followed by the application of DDD as described by Barrnett and Seligman (1954).

Counts of the follicle density (number per unit area) were made with a microprojector at a magnification of $\times 215$ on four to six fields, each 1 mm^2 in area at the mid-sebaceous gland level. The counts were corrected for shrinkage in a manner similar to that described by Carter and Clarke (1957). The following counts were recorded: number of primary (P) plus secondary (S) follicles per mm^2 , number of P follicles per mm^2 , and the S/P ratio.

Sections from superficial levels were also examined to study the thickness of the epidermis and at a deeper level to study the histology of the sweat glands.

III. RESULTS

Biopsies of the cysts (see Table 1) were made at intervals and entire cysts were removed for examination 42–83 weeks after implantation. All cysts increased in diameter; the surface area of the largest had increased more than four times when removed at 83 weeks. In some cases the first measurements of cyst diameter were less than the diameter of the semicircular sac implanted. This can be explained by (1) the initial contraction of skin when released from its usual state of tension; or (2) the inflation of the implanted skin by cyst contents before growth occurred.

Examination of the cyst wall removed at biopsy showed that burying sheep-skin had apparently not affected either the structure or the function of wool follicles, sebaceous glands, or sweat glands. Established cysts contained wool fibres and fluid, and volumes up to 12 ml were aspirated. In some cases the syringe plunger was forced back when aspirating the fluid contents. Plate 2, Figure 1, shows sweat glands lined by active epithelium in a cyst wall 83 weeks after implantation.

TABLE 1

TIMES OF SAMPLING AND SIZE OF CYSTS

Measurements of two diameters are given in centimetres. S, biopsy of cyst and control skin; R, cyst removed

Breed	Cysts Implanted	Time (weeks) after Implantation										
		5	8	13	18	23	28	42	47	55	78	83
Merino B432	A			S 2.8 by 2.2	3.2 by 2.5		S 3.5 by 2.7		S 5.0 by 3.7			SR 6.0 by 4.7
	B	S		S 2.7 by 2.2	2.8 by 2.2		S 3.2 by 2.2		S 4.2 by 3.0			SR 5.2 by 4.0
Merino B195	A		S 4.0 by 2.4	S 4.4 by 2.5		S 5.5 by 3.2		S 6.5 by 3.7			SR 6.4 by 4.1	
	B			S 4.2 by 2.2		S 4.8 by 2.5		SR 6.5 by 4.0				
	C							S 5.5 by 3.0			SR 5.3 by 4.0	
Merino A445 (sweat glandless mutant)	A			S 2.2 by 2.2	S 3.5 by 2.7					SR 4.0 by 3.0		
Corriedale A459	A	S		S			S 4.0 by 3.2		SR 5.5 by 4.0			

To assess the effect of the cyst environment on the function of wool follicles, the rate of wool growth in the Corriedale cyst was determined by the method of Downes and Lyne (1959) using [^{35}S]cystine. Forty-seven weeks after implantation the rate of growth of the cyst wool, 0.46 mm per day, was similar to that of surface wool.

The distribution of sulphhydryl and disulphide bonds corresponded with that of control sections, indicating that normal keratinization probably occurred in the cyst environment (Molyneux, unpublished data).

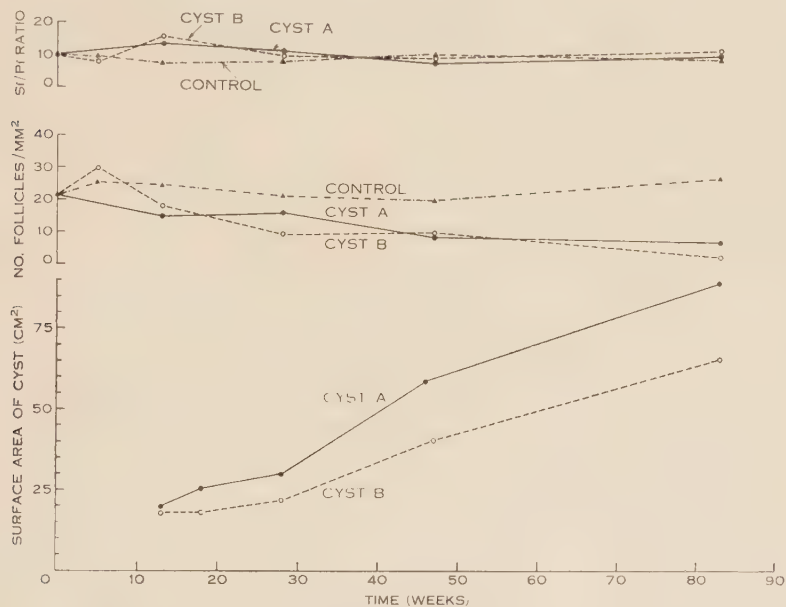


Fig. 1.—Relation between time after implantation, surface area of cysts, number of follicles per mm², and secondary/primary fibre (Sf/Pf) ratio in control and cyst samples in Merino B432.

Subsequent to the implantation and biopsy procedures it was expected that inflammatory changes would be present in the cyst walls. However, inflammatory reaction was slight and was restricted to a round cell infiltration immediately beneath the epidermis. This infiltration gradually disappeared as the time between biopsies was increased.

Up to 83 weeks after implantation no structural alteration in any of the epidermal derivatives of cyst skin had occurred as compared with surface skin. However, the arrangement of the follicle groups had altered considerably. For example, Plate 3, Figure 1, shows a transverse section of surface skin at the mid-sebaceous gland level in which the follicles are arranged in groups as described by Carter (1943). Plate 3, Figure 2, shows a similar section of cyst wall. Follicles are still grouped together but the groups are further apart as a result of an increase in the major trabeculae of connective tissue in which arrector pili muscles can be clearly seen. Within the group there is evidence that the primary follicles tend to become

isolated while the secondary follicles form subgroups as minor trabeculae of connective tissue increase in width. The outstanding features are the increase in connective tissue trabeculae and the absence of inflammation. To demonstrate this increase in connective tissue, diameters of follicle groups and distances between adjacent groups (measured from the centre of each follicle group) were recorded. For example, the average distance between 15 groups in a control sample of surface skin from animal B432 was $1030\ \mu$ and the average diameter was $850\ \mu$. In cyst A

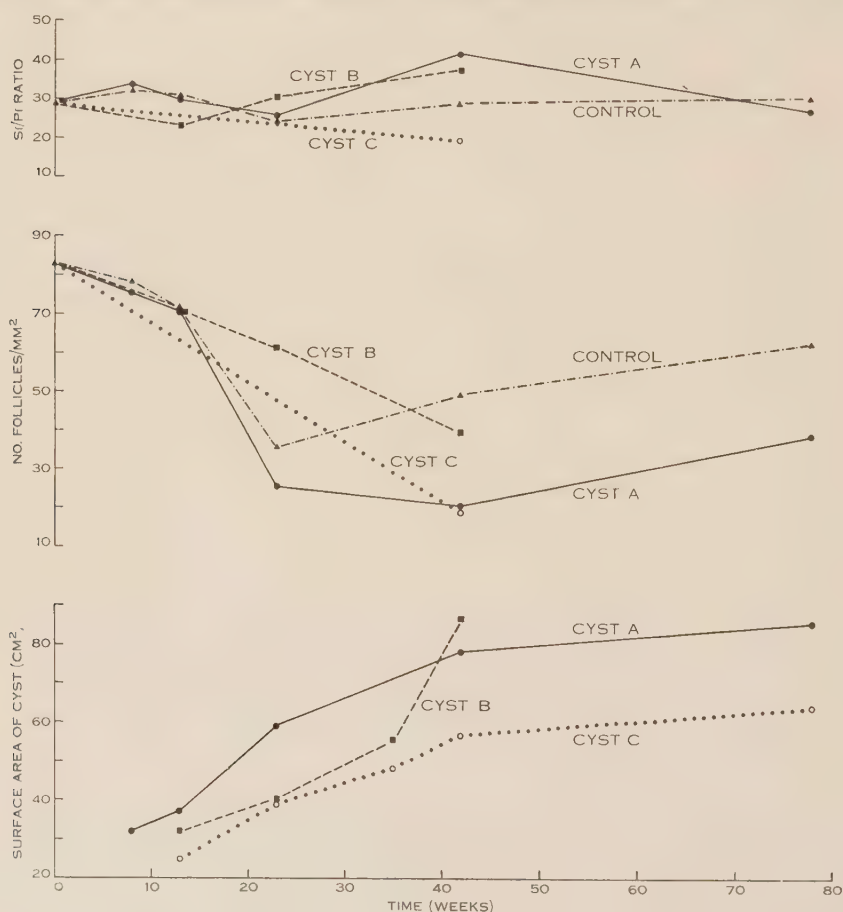


Fig. 2.—Relation between time after implantation, surface area of cysts, number of follicles per mm^2 , secondary/primary fibre (Sf/Pf) ratio in control and cyst samples in Merino B195.

of the same sheep 83 weeks after implantation, the average distance between four groups was $1480\ \mu$ and the average diameter was $1090\ \mu$. Only a few satisfactory measurements could be made on the cyst samples because of their small diameter ($0.5\ \text{cm}$), the wide separation of their groups, and the difficulty of unequivocally defining the outlines of follicle groups.

Examination of the cyst samples below the sebaceous gland level showed the presence of bud-like structures originating from the outer root sheath. They occurred

at a level just above the dermal papilla of the follicle bulb and multiple structures were seen to originate from a single follicle (Plate 2, Fig. 2). The larger structures resembled small cysts attached by a stalk to the outer root sheath and having a lumen lined by tissue similar to the cornified layer of epidermis. Similar structures have been described by Burns and Clarkson (1949), Auber and Ryder (1955), and Epstein and Kligman (1956). Examination of biopsy samples showed that these structures were present in all the control samples of all the sheep and that their occurrence was apparently more frequent in the cyst wall. This apparent increase in frequency may depend upon the large number of follicles in the cyst samples which have been cut obliquely or longitudinally, thus enabling the structures to be more readily seen.

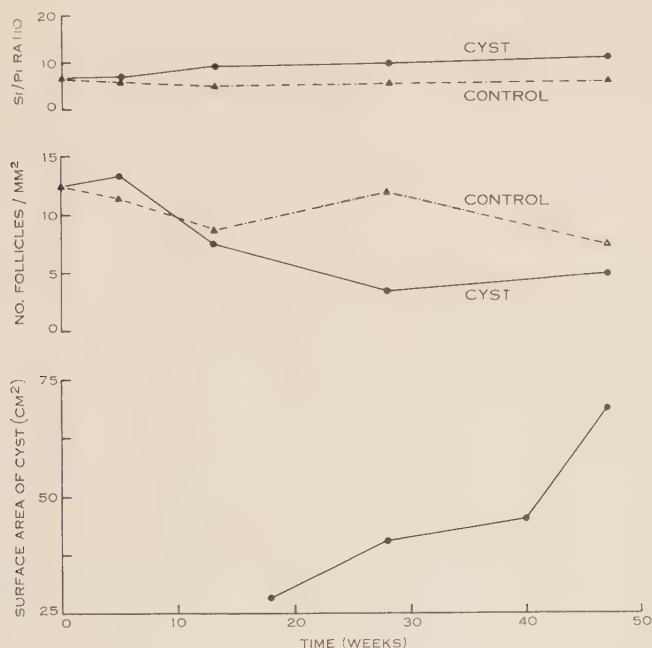


Fig. 3.—Relation between time after implantation, surface area of cyst, number of follicles per mm², and secondary/primary fibre (*Sf/Pf*) ratio in control and cyst samples in Corriedale A459.

The number of *P* plus *S* follicles per mm² and *S/P* fibre ratios (*Sf/Pf*) for each skin sample, together with the changes in surface area of the cysts, are shown in Figures 1-4.

The surface area of all cysts in all animals increased with time. In animals B195 and B432 multiple implantations were made and the growth of the resulting cysts observed for 78 and 83 weeks respectively. Although individual cysts in the same animal did not attain the same size, their growth curves indicated a similar growth pattern (Figs. 1 and 2). The least growth occurred in the Merino mutant without sweat glands. In general, the rate of growth tended to decrease with time.

The density of the follicles in the cyst samples decreased as the age of the cyst increased. The low incidence of shed or abnormal follicles in the cyst skin when compared with control skin indicated that the decrease in density was the result of an increase in surface area, which was dependent upon an increase in connective tissue between follicle groups and an increase in the epidermis. The smallest decrease in follicle density occurred in the Merino mutant without sweat glands. In some cases there was an initial rise in follicle density which was considered to be the result of skin contraction.

Because of the low incidence of shed or abnormal follicles the *S/P* fibre ratios are equivalent to the *S/P* follicle ratios. The constancy of the *S/P* ratios in the cyst and control samples supports the conclusion that the decrease in follicle density

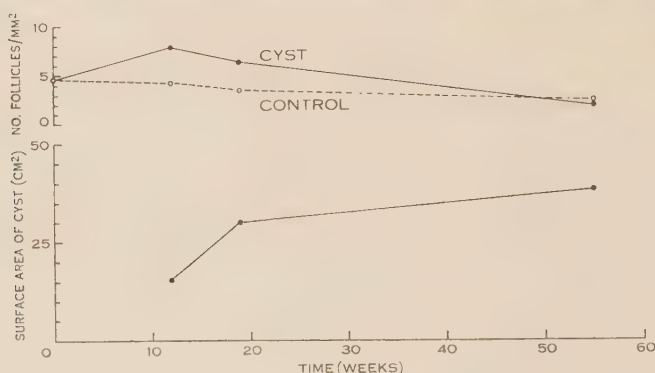


Fig. 4.—Relation between time after implantation, surface area of cyst, and number of follicles per mm² in control and cyst samples of Merino mutant without sweat glands, A445.

was mainly the result of the separation of follicle groups. The apparent increase in the *S/P* ratio in the Corriedale cyst samples was thought to be due to an inadequate number of follicle groups in such small samples in an animal with such a low follicle density. Because of the absence of sweat glands in animal A445 no attempt was made to identify the primary follicles. Thus no *S/P* data appear in Figure 4.

IV. DISCUSSION

The sheep has proved to be an ideal experimental animal for the study of cyst growth. Knowledge of the biology of sheepskin has enabled the wool follicles to be used as growth markers to reflect the influences of the environment.

To study growth in an artificially induced biological system, such as an experimental dermal cyst, the system must be reproducible if data are to be interpretable in physiological terms. Although the sheep chosen provided a wide range of skin characteristics, the similar behaviour of all cysts indicates that the implantation technique can be used successfully to form standard epithelium-lined cysts which will follow a predictable growth pattern; this is taken as evidence of the stability of the cyst system. Results showed that increase in surface area of cysts was related to their age, and that the growth rate gradually decreased with time.

As expected, the growth of cysts was accompanied by a decrease in wool follicle density per unit area. This decrease in density is apparently not the result of loss of follicles but rather a separation of follicle groups by growth of interstitial connective tissue. This view is supported by the apparent constancy of the S/P ratio and particularly by the absence of shed or abnormal follicles in the cyst wall as compared to the control skin samples.

It was found in the cysts on the sheep without sweat glands that after 55 weeks only slight growth had occurred and that the follicle density had fallen only slightly below that of the control skin. It would thus seem that factors contributing to cyst contents, such as secreting epithelium or inflammatory exudate, are probably important in determining the rate of growth and eventual size of such cysts.

The absence of abnormalities in either wool follicles or their accessory structures, the normal growth rate of intracystic wool, and the similar distribution of sulphhydryl and disulphide bonds in the cyst wall to that in surface skin, all indicate that the cyst environment causes no detectable change in the metabolism of the implanted skin. Although the apparent absence of structural or metabolic change suggests that the cyst wall is not subjected to pressure, the forcing back of the syringe plunger when aspirating fluid cyst contents and the spherical shape assumed by cysts indicate the presence of positive intracystic hydrostatic pressures. It is probable that the intracystic pressure is balanced by the active growth of connective tissue and an increase in the local blood pressure within the cyst wall.

The selection of animals of different breeds and with specific genetic characteristics can extend the scope of these artificially induced cysts as a biological tool. For example, the potassium content of cyst fluid from an animal with sweat glands was 121 m-equiv/l. This value agrees with the potassium concentration of sweat collected by other methods and the potassium content of cyst fluid from an animal without sweat glands was 9.2 m-equiv/l, which is similar to the potassium value for extracellular body fluids (Short, Stacy, and Brook, personal communication).

Because cyst skin is similar in structure and metabolism to surface sheepskin and because it would be possible to maintain a high antibiotic concentration in the protected cyst environment over an extended period, these cysts could be used as a source of sterile adult skin and sterile wool. The use of experimental dermal cysts to produce artificially the environment necessary for the development of "pink rot" has already been discussed (Molyneux 1959).

The establishment of these cysts as a reliable biological system suggests their use in studies of skin metabolism and also will enable the study of cyst growth to be continued.

V. ACKNOWLEDGMENTS

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One of us (G.S.M.) is also indebted to the National Health and Medical Research Council for a grant to enable this work to be carried out.

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EXPLANATION OF PLATES 1–3

PLATE 1

- Fig. 1.—Experimental cyst removed from Merino sheep without sweat glands 55 weeks after implantation ($\times 3$).
- Fig. 2.—Experimental cyst removed from Merino sheep with sweat glands 83 weeks after implantation ($\times 3$).

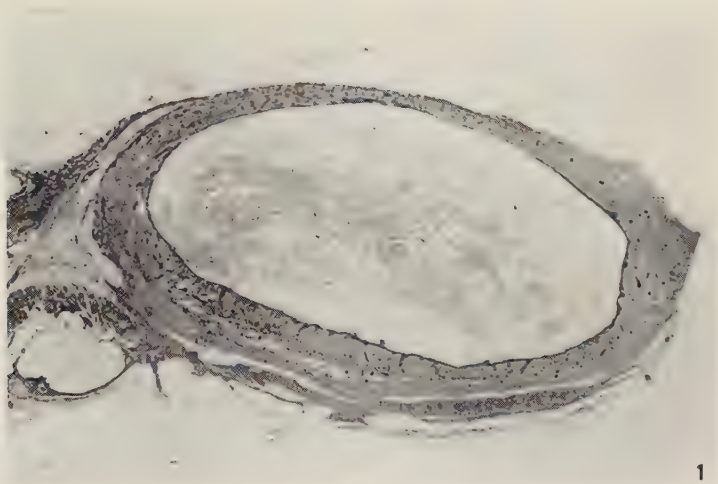
PLATE 2

- Fig. 1.—Photomicrograph of cyst wall from Merino sheep showing sweat glands with active epithelium 83 weeks after implantation ($\times 160$).
- Fig. 2.—Photomicrograph of wool follicle in cyst wall 83 weeks after implantation showing two cyst-like structures arising from the outer root sheath of a single follicle ($\times 180$).

PLATE 3

- Fig. 1.—Section showing group arrangement of follicles in control skin sample of Merino B432 ($\times 20$ approx.).
- Fig. 2.—Section showing group arrangement of follicles in cyst skin from Merino B432, 83 weeks after implantation ($\times 20$ approx.). *M*, major connective tissue trabecula separating follicle groups; *m*, minor connective tissue trabecula dividing follicle group into subgroups; *P*, primary follicles isolated from follicle group; *A*, arrector pili muscle in major trabecula.

DERMAL CYSTS IN SHEEP

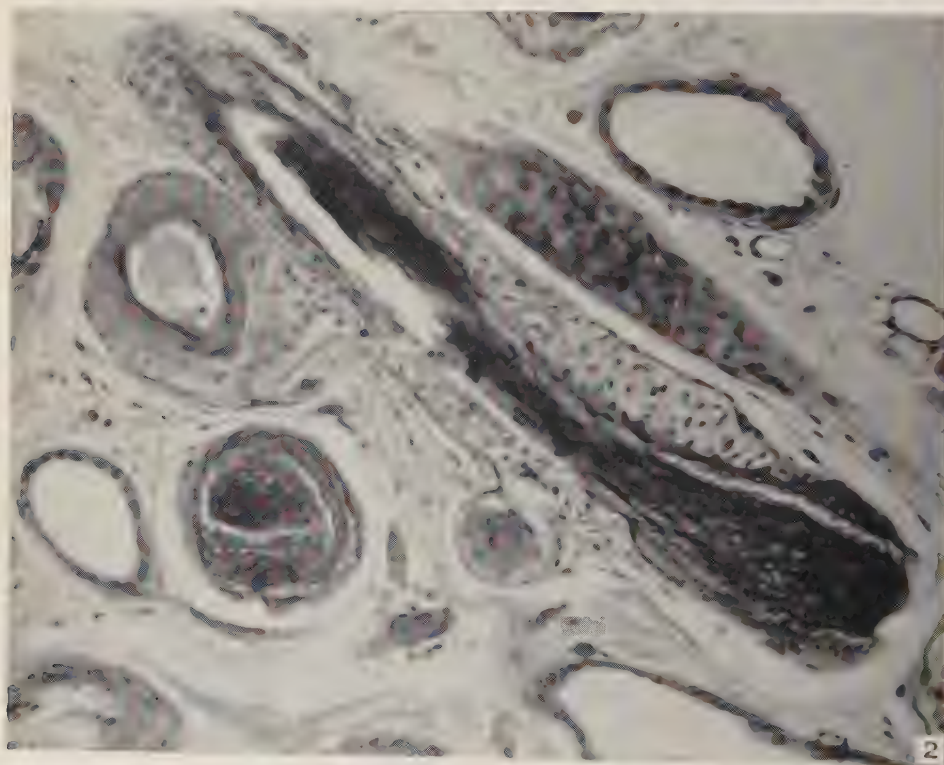
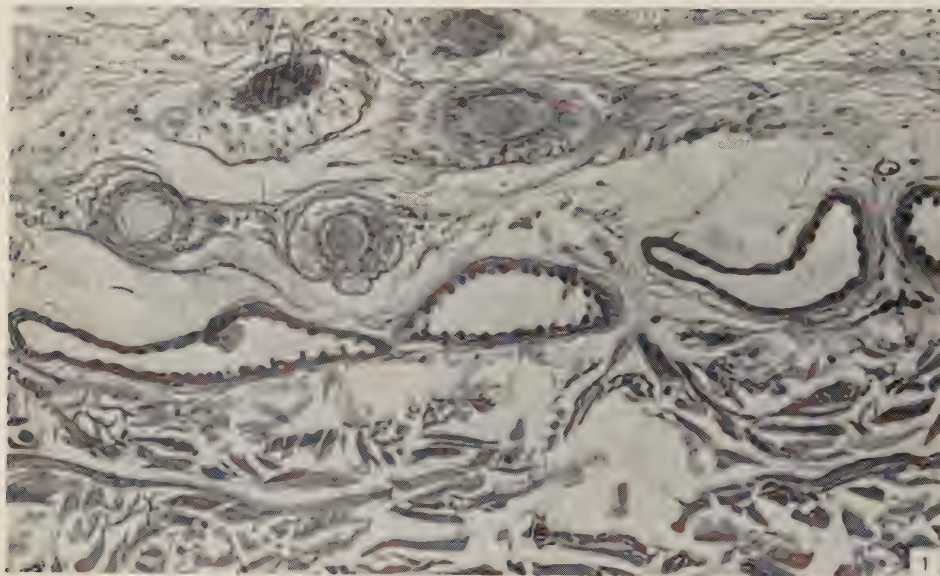


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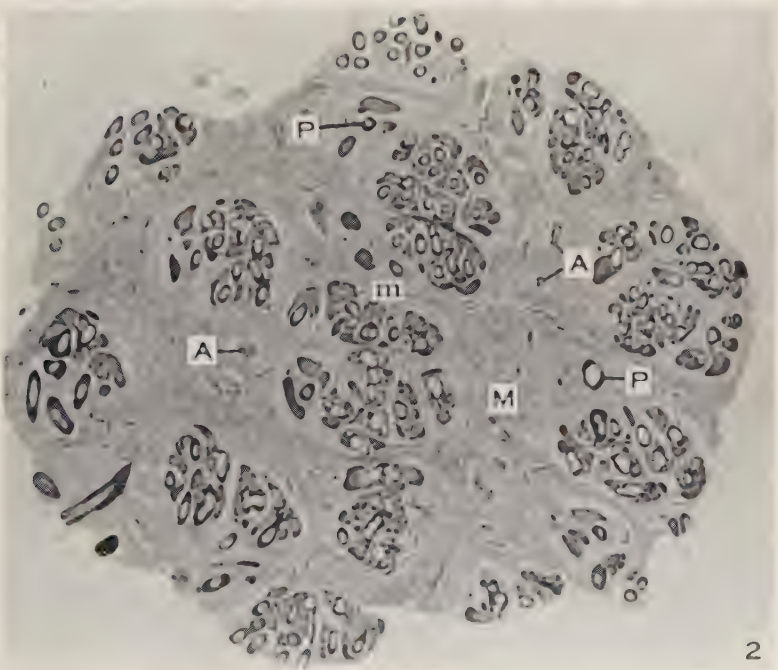
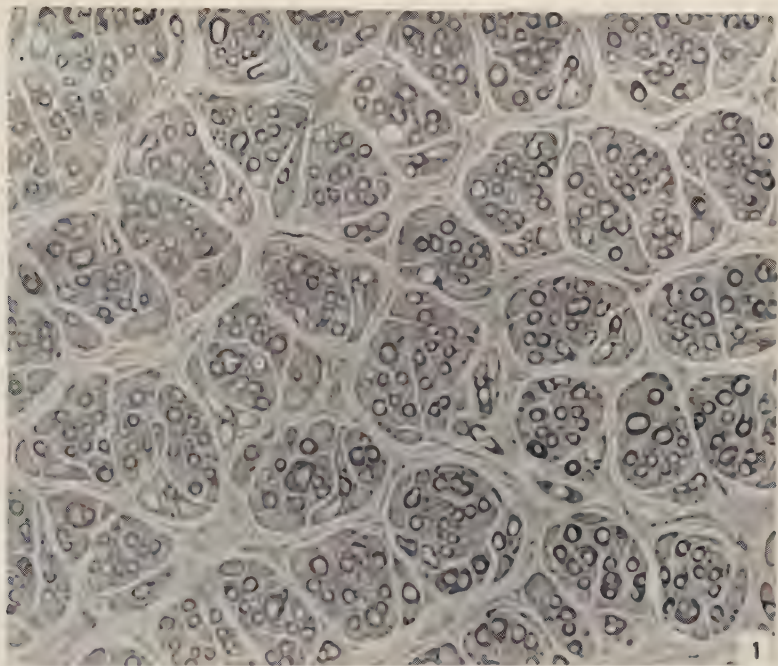


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DERMAL CYSTS IN SHEEP



DERMAL CYSTS IN SHEEP



THE POSTNATAL DEVELOPMENT OF WOOL FOLLICLES, SHEDDING, AND SKIN THICKNESS IN INBRED MERINO AND SOUTHDOWN-MERINO CROSSBRED SHEEP

By A. G. LYNE*

[Manuscript received September 16, 1960]

Summary

A quantitative study has been made of the postnatal development of the follicle population, the incidence of shed follicles, and changes in skin thickness in four Merinos and four Southdown-Merino crossbreds. The animals were sampled on 30 occasions from birth to approximately 2 years.

In addition to the two main types of follicles—primary (*P*) and secondary (*S*)—a third type, termed primo-secondary (*PS*) follicles, has been recognized. These follicles are usually situated on the ectal margin of the follicle group and possess associated sweat glands; arrector pili muscles are seldom present.

Most of the immature follicles (*Si*) seen in birth and early postnatal samples were derived secondaries (*SD*). As these follicles arise by branching, either from the original secondary (*SO*) follicle or from other derived secondary follicles, it is usually impossible to include all of them in counts made at only one level in the skin.

In general the $S/(P+PS)$ follicle ratios obtained for the early samples (birth to 60 days) were similar to or lower than they were for later samples.

In both the Merinos and crossbreds the incidence of shed follicles was extremely low. In the Merinos, shed follicles varied from a maximum of 1.4% in one animal at 631 days to 8.5% in another animal at the same age. In the crossbreds, shed follicles varied from 1.1% in an animal at 445 days to 12.1% in an animal at 277 days. No marked influence of season on the incidence of shed follicles was discernible.

There was no trend of skin thickness with age except during the early postnatal period when most sheep showed a slight upward trend. Other factors which appeared to influence the thickness were nutrition, pregnancy, and shearing. The skin was thicker in the crossbreds than in the Merinos.

I. INTRODUCTION

Although the general features of the development and growth of wool follicles are now well established, little attention has been given to the detailed histological changes in follicles that take place from birth to adulthood. Such studies are important for a better understanding of the adult fleece and the control of wool growth.

This paper is an account of the postnatal development of the follicle population, the incidence of shed follicles, and age and seasonal changes in skin thickness in four Merino and four Southdown-Merino crossbred sheep. These quantitative

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observations formed part of detailed histological studies of the formation of bundles of primary and secondary follicles, to be described in a subsequent paper.

II. MATERIAL AND METHODS

(a) *Animals*

Four inbred Merinos (2 ewes and 2 wethers), born 7-10.x.56, and four Southdown-Merino crossbreeds (3 ewes and 1 wether), born 29.ix.56-1.x.56, were used for this study. All the sheep were born and reared in the field at the C.S.I.R.O. McMaster Field Station, Liverpool, N.S.W. Each animal was weighed at weekly or fortnightly intervals from birth to approximately 2 months, and at irregular intervals thereafter. All animals were shorn at about 130, 305, and 675 days. Three of the ewes lambed twice and the remainder once during the experimental period. Four of them first became pregnant at 216-229 days, with subsequent lambing and lactation.

(b) *Skin Sampling and Histological Methods*

On 30 occasions from birth to approximately 2 years, two skin samples were taken with a 1-cm trephine from the mid-lateral region of the trunk of each sheep. The samples were taken from one side of the trunk up to sample 15 when sampling of the other side was commenced and continued to sample 30. Samples 15, 18, and 30 were taken from both sides of the body.

The mean ages at the second and later sampling were as follows: Southdown-Merinos—17, 31, 45, 66, 87, and 108 days, and at 28-day intervals to 752 days. Merinos—8, 22, 36, 57, 78, and 99 days, and at 28-day intervals to 743 days. One Merino died after the 28th sampling.

All the skin samples were fixed in 5% formol saline. One of the duplicate samples taken on 19 different occasions (Nos. 1-5, 7, 9, 11, 13, 15 (both sides), 16, 18 (both sides), 19, 21, 23, 25, 26, 28, and 30 (both sides)) were serial-sectioned at 8μ parallel to the skin surface, and from the skin surface to the lower sebaceous gland level. A few of the remaining samples, taken on the same occasions, were serial-sectioned at 8 and 15μ at right angles to the skin surface. Staining was with haemalum, eosin, and picric acid.

(c) *Skin and Follicle Measurements*

(i) *Macroscopic*.—The thickness of at least one of the skin samples (excluding the *panniculus carnosus*) was measured after fixation with an instrument (Wodzicka 1958a) which exerted a constant pressure of 50 g/cm^2 . The wool on each sample was closely clipped.

(ii) *Microscopic*.—Using a microprojector and a magnification of $\times 215$, counts of the follicle and fibre density on transverse sections of skin (i.e. sections parallel to the skin surface) at the mid-sebaceous gland level were made on six circular fields of 1 mm^2 . The counts were corrected for shrinkage in a manner similar to that described by Carter and Clarke (1957). A small spot of Indian ink was placed on the coverslip above the section selected for counting. This spot was then used

as a "landmark". By this technique every field was relocated easily for re-examination and for more detailed observations with a research microscope.

(d) Terminology

The terminology of Hardy and Lyne (1956a) is used with the addition of primo-secondary (*PS*) follicles. These follicles, usually situated near the ectal margin of the follicle group, have sweat glands which are smaller than those of primary (*P*)

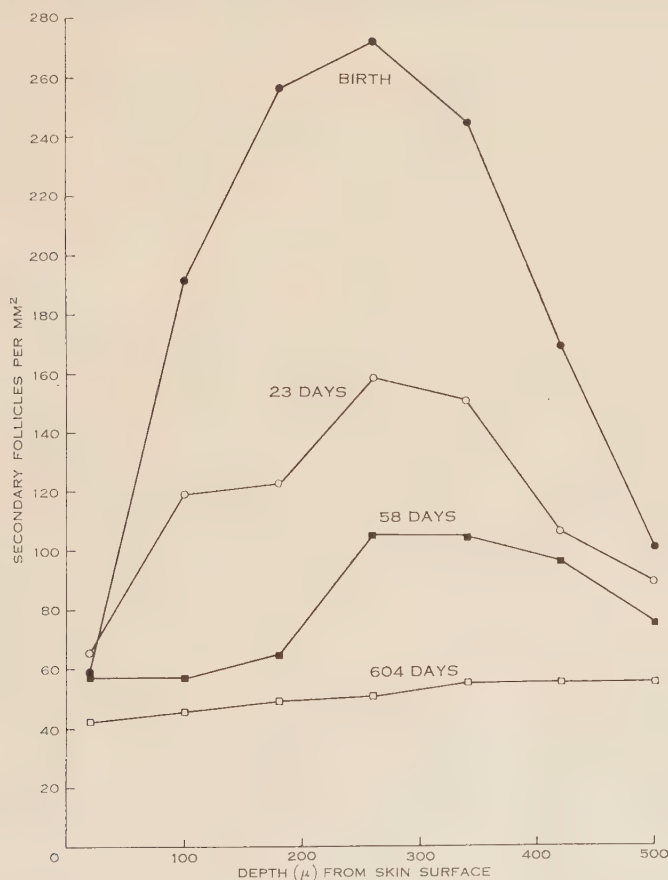


Fig. 1.—Relation between number of *S* follicles per mm² and depth from the skin surface in four skin samples from one inbred Merino (K293). Each point is the mean of counts of four 1-mm² fields.

follicles, and are intermediate in size between *P* and secondary (*S*) follicles. Arrector pili muscles are seldom present. *PS* follicles have been included with *P* follicles except where otherwise indicated. Follicles belonging to bundles of apparent *P* follicles with a single associated sweat gland (Lyne 1957) were also included in the primary follicle count.

The suffix *e* to follicle type symbols denotes a *dormant* follicle which has lost its fibre.

III. THE DEVELOPMENT OF THE FOLLICLE POPULATION

(a) Relation between Apparent Number of Secondary Follicles per Unit Area and Depth

To determine the level in the skin at which there is a maximum density of immature secondary (*Si*) follicles, the number of follicles in serial sections of samples, taken at birth, 23, 58, and 604 days, from one sheep, were counted. The sample taken at 604 days was included for comparison but did not contain immature follicles.

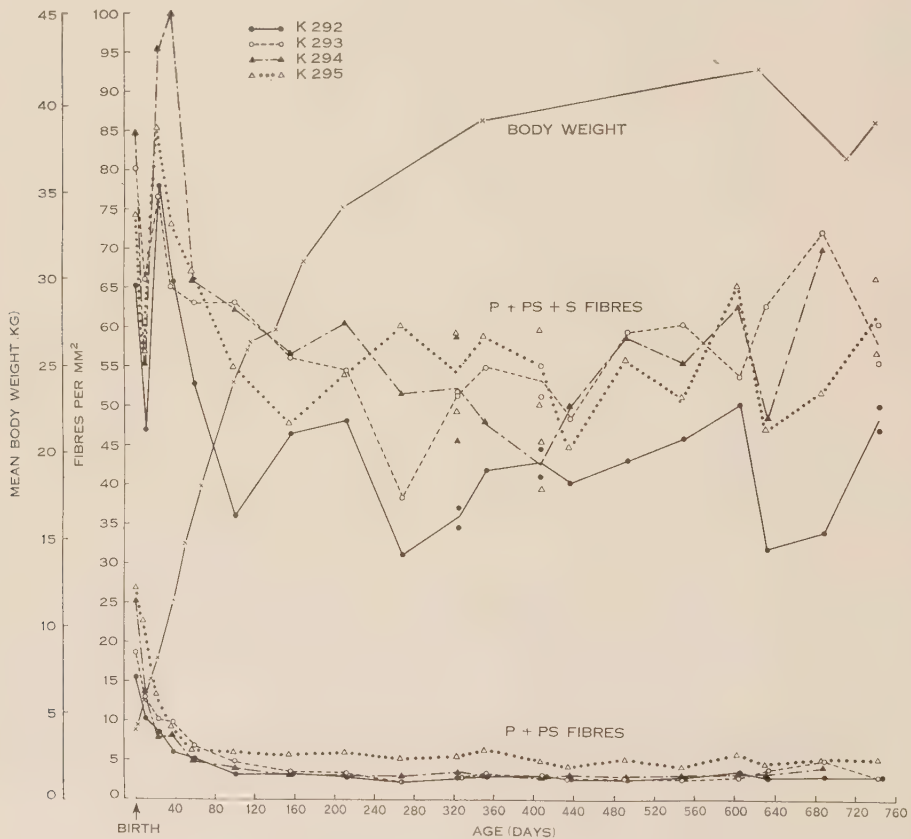


Fig. 2.—Relation between age, mean body weight, and fibre density in four inbred Merinos. Where counts were made on samples from both sides of the body, the lines are drawn midway between the values.

These counts showed that at each age the maximum density was reached at about the same depth (250–300 μ from the skin surface) but that the peak of density was much sharper at birth than it was at 23 or 58 days (Fig. 1). Therefore, the depth of counting immature follicles in the new-born lamb must be selected with much greater care than when dealing with older material. The level showing the maximum density of follicles was close to the mid-sebaceous gland level at which all the other measurements were made.

(b) Primary and Secondary Follicles and Fibres per Unit Area

The changes in the fibre population density (number of $P+S$ fibres per mm^2) for each sheep during the postnatal period studied are shown in Figures 2 and 3. The density of the P fibres for each sheep is also shown in these figures.

In the Merinos (Fig. 2) the decrease in $P+S$ fibre density during the first week after birth was followed by a rapid rise and fall. This feature was not seen in the crossbreds (Fig. 3), probably due to the longer interval between the first and

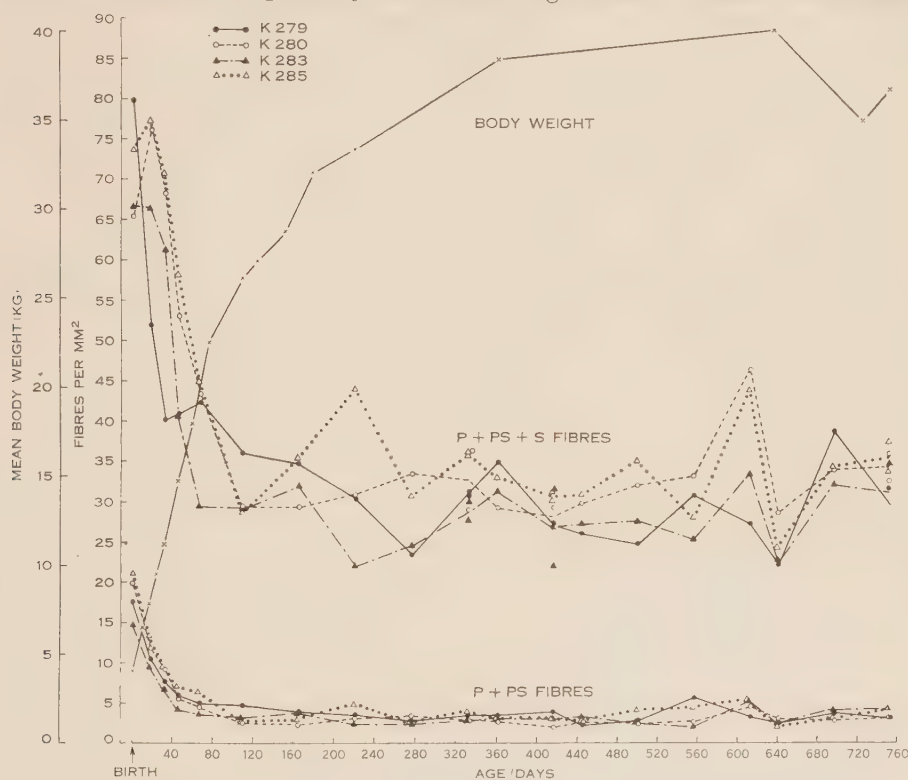


Fig. 3.—Relation between age, mean body weight, and fibre density in four Southdown-Merinos. Where counts were made on samples from both sides of the body, the lines are drawn midway between the values.

second sampling. After 3–4 months the density of each genotype remained fairly constant up to 2 years of age.

Comparison of Figures 2, 3, and 4 shows that the Merinos had a higher follicle and fibre density than the crossbreds. The only exception was the second sample in which the crossbreds had a higher fibre density than the Merinos (Fig. 4).

Although the mean fibre population densities of the two genotypes were similar at birth (about 75 per mm^2 for the Merinos and 71 per mm^2 for the crossbreds) they were distinctly different after all the follicles had matured, mainly because the densities in the crossbreds decreased by more than 100% and in the Merinos by only about 50%.

The follicle population density (Fig. 4) decreased rapidly during the first 3–4 months. For the Merinos the mean number of $P+S$ follicles at birth was about 290 per mm^2 compared with about 180 per mm^2 for the crossbreds. By 110 days in the crossbreds and 155 days in the Merinos most of the S follicles had matured, hence the fibre and follicle densities became almost equal. At all ages the follicle density for the Merinos was above that for the crossbreds. Considering only samples taken after 5 months of age, the mean follicle density for the Merinos is 51.8 per mm^2 , and for the crossbreds it is 31.5 per mm^2 . For individual animals, the means and standard deviations for the same period are shown in Table 1.

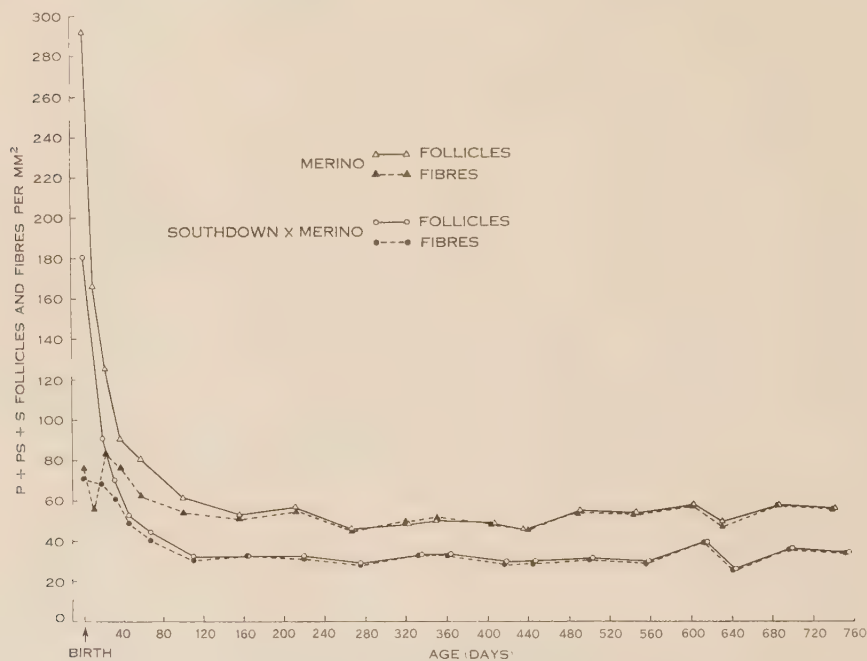


Fig. 4.—Relation between age and mean follicle and fibre density in four inbred Merinos and four Southdown-Merinos.

All the P follicles had matured (i.e. they contained a keratinized fibre) at birth. P follicle density, similar in the two genotypes at birth, also decreased rapidly during the early postnatal period (Figs. 2 and 3). The apparently higher density of P follicles in one Merino was due to a high proportion of PS follicles.

(c) Secondary/Primary Follicle and Fibre Ratios

The S/P follicle ratios for each sheep during the postnatal period studied are shown in Figures 5 and 6. In general, the follicle ratios obtained for the early samples (birth to 2 months) are similar to or lower than they are for later samples. In the Merinos the mean follicle ratios obtained for the first five samples compared with the last five samples (Table 1) show an increase of from 13.2 to 35.9%. The actual number of S follicles in the early samples must be higher than the results

obtained because the counting was done at only one level. In contrast to the Merinos, only one of the crossbreds shows a large increase (29%) in the mean follicle ratio when these two periods are compared. Of the remainder, two show no change and one shows a small decrease (7.4%).

The $S/(P+PS)$ follicle ratios were generally higher in the Merinos than in the crossbreds (Table 1). The one exceptionally low ratio in the Merinos was due to the high proportion of PS follicles.

TABLE 1

COMPARISON OF THE $S/(P+PS)$ FOLLICLE RATIOS AND $P+PS+S$ FOLLICLE DENSITIES IN FOUR INBRED MERINOS AND FOUR SOUTHDOWN-MERINO CROSSBREDS

Genotype	Sheep No. and Sex	$S/(P+PS)$ Follicle Ratios						$P+PS+S$ Density	
		First 5 Samples Examined (birth-2 months)		Last 5 Samples Examined (17-24 months)		Last 13 Samples Examined (5-24 months)		Last 13 Samples Examined (5-24 months)	
		Range	Mean	Range	Mean	Mean	S.D.	Mean	S.D.
Inbred Merino	K292♂*	8.5-12.8	11.1	10.4-15.0	12.7	13.3	± 1.82	41.8	± 6.3
	K293♂*	8.5-14.6	13.1	13.2-22.2	17.8	17.2	± 2.14	55.6	± 6.9
	K294♀	11.6-15.3	13.3	13.8-18.6	16.4	15.8	± 1.76	53.5	± 8.2
	K295♀	7.6-10.4	9.1	8.9-12.1	10.3	9.8	± 1.02	56.0	± 6.1
Southdown-Merino	K279♀	6.1-8.9	7.6	4.5-9.1	7.5	7.6	± 1.33	30.0	± 4.3
	K280♀	6.0-9.0	7.9	8.7-11.9	10.2	10.4	± 1.12	32.8	± 4.7
	K283♀	7.1-9.3	8.1	4.7-11.8	7.5	8.0	± 1.72	28.8	± 4.2
	K285♂*	7.0-9.6	8.0	5.3-10.9	8.1	8.7	± 1.58	34.4	± 5.1

* Wethers

Special attention was given also to the follicle ratios in one of the crossbreds (K285) which had a high frequency of PS follicles (Fig. 7). In samples taken after 5 months of age the mean $S/(P+PS)$ ratio is 8.7 ± 1.6 , whereas the mean $(S+PS)/P$ ratio is 10.3 ± 2.0 .

At birth the mean S/P fibre ratios in the two genotypes were very similar; 2.7 for the Merinos and 3.0 for the crossbreds. Figure 8 shows the percentage of mature S follicles in each sheep from birth to about 1 year. The difference in the percentage of mature S follicles in the two genotypes at birth (17-25% for the Merinos; 25-44% for the crossbreds) was apparently due to the fact that a higher percentage of SD follicles occurs in the Merinos. In both genotypes the maximum rate of S follicle maturation was between birth and 1 month, and this is in agreement with the observations of Schinckel (1955a) and Short (1955b) for other Merinos and Merino crossbreds. The follicle population as a whole appeared to mature earlier in the crossbreds than in the Merinos.

Pregnancy and lactation doubtless added to the variability of the mature follicle ratios and densities. These effects, however, were subsequent to the phases of the development of the follicle population considered here.

IV. SHEDDING

The number of *dormant* follicles which had shed and released their fibres was also counted in each of the six 1-mm² fields. As shedding was sometimes localized

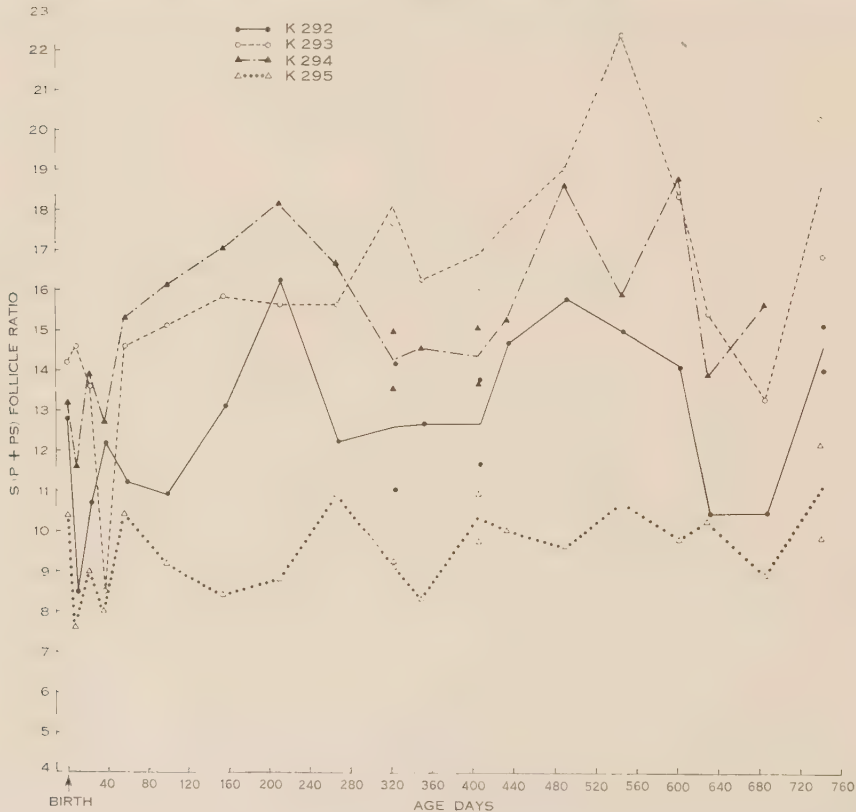


Fig. 5.—Relation between age and $S/(P+PS)$ follicle ratio in four inbred Merinos. The low ratio in one animal is due to the high frequency of *PS* follicles. Where counts were made on samples from both sides of the body, the lines are drawn midway between the values.

these counts did not always give a satisfactory estimate of the abundance of the shed follicles in the whole trephined sample. Because of this the *approximate* number of shed follicles over the whole sample was counted and scored as follows: 0 = no shed follicles; 1 = 1–5 shed follicles—very little shedding; 2 = 6–15 shed follicles—little shedding; 3 = 16–25 shed follicles—some shedding; 4 = >25 shed follicles—shedding common.

(a) *Relation between Follicle Type and Shedding*

Tables 2 and 3 summarize the observations and it is clear that the incidence of shed *P* and *S* follicles was extremely low; in fact, for all practical purposes shedding was negligible.

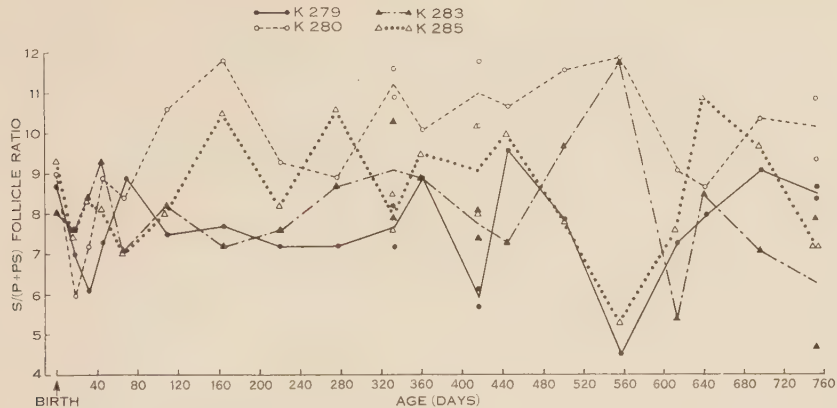


Fig. 6.—Relation between age and $S/(P+PS)$ follicle ratio in four Southdown-Merinos. Where counts were made on samples from both sides of the body, the lines are drawn midway between the values.

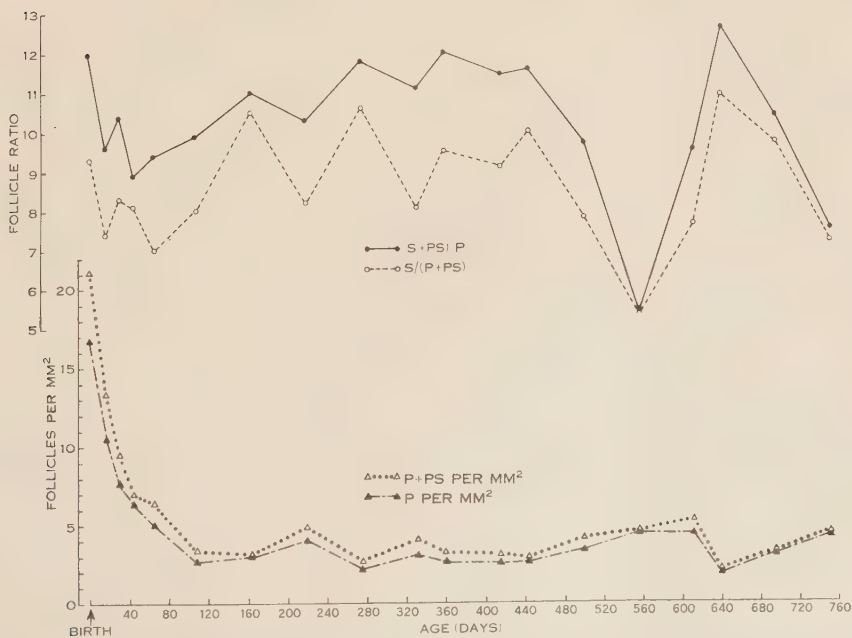


Fig. 7.—Relation between age, follicle ratios $S/(P+PS)$ and $(S+PS)/P$, and density of primary follicles (*P* and *P+PS*) in one Southdown-Merino (K 285).

In the Merinos (Table 2) there were little, if any, differences between the percentage of shed *P* and *S* follicles. It is interesting to note that in one sheep there

were no *Pe* or *PSe* follicles in a total of 981 examined. There were, however, a few shed primary follicles outside the fields examined.

In the crossbreds (Table 3) *Se* follicles were fewer than *Pe* follicles.

(b) *Relation between Age, Season, and Shedding*

The percentage of shed follicles (*Pe* + *PSe* + *Se*) in each sample has been plotted against age (Fig. 9) to reveal possible age or seasonal trends in the frequency of shedding.

In general, the proportion of shed follicles increased with age. While all samples taken from birth to about 160 days exhibited less than 1.5% of shed follicles, in

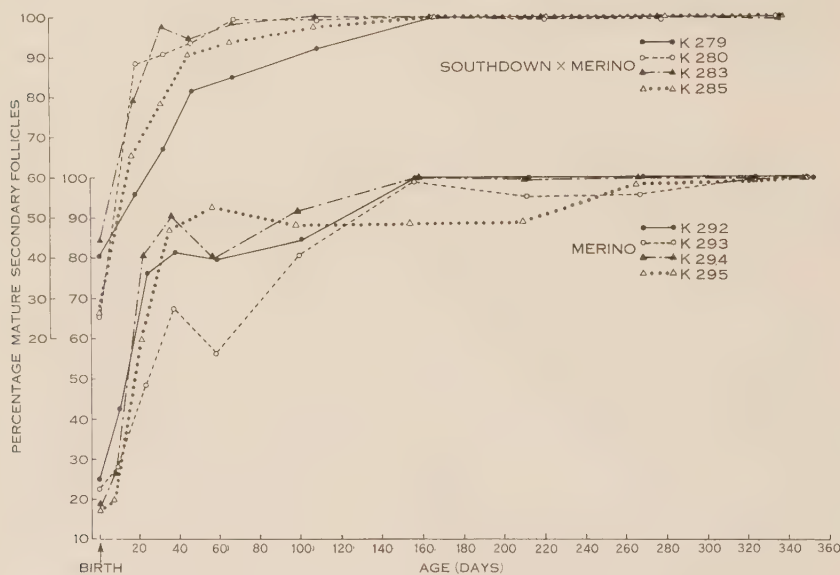


Fig. 8.—Percentage of mature *S* follicles in four inbred Merinos and four Southdown-Merinos from birth to 1 year.

the Merinos the maximum percentage of shed follicles was only 1.4% in one animal at 631 days and 8.5% in another at 630 days. Amongst the crossbreds shed follicles varied between a maximum of 1.1% for one animal at 445 days to 12.1% for another at 277 days.

No marked influence of season on the incidence of dormant follicles without fibres was discernible.

The shedding score, being based on an examination of the whole sample, is probably a better indication of the true frequency of shed follicles than the percentages which referred only to the particular six fields examined. There appeared to be a trough, both in percentage and shedding score, in the late spring-early summer for the Merinos at the beginning of their second year (Fig. 9), but the data were not extensive enough to confirm or confute this in a later year.

V. CHANGES IN SKIN THICKNESS

The changes in mean skin thickness for each genotype are shown in Figure 10. The mean skin thickness of the crossbreds was always higher than that of the Merinos,

TABLE 2
SUMMARY OF OBSERVATIONS ON FIBRE SHEDDING IN FOUR INBRED MERINOS

Sheep No.	Age Period Examined	Total No. of <i>P+PS</i> Follicles Examined	<i>Pe+PSe</i> Follicles*		Total No. of <i>S</i> Follicles Examined	<i>Se</i> Follicles*	
			No.	% of Total <i>P+PS</i>		No.	% of Total <i>S</i>
K292	Birth-745 days	826	9	1.09	10,075	63	0.59
K293	Birth-744 days	963	5	0.52	14,404	38	0.26
K294	Birth-687 days	981	0	0	14,127	29	0.21
K295	Birth-742 days	1452	9	0.62	13,648	115	0.84
Total *		4222	23	0.54	52,254	242	0.46

* Dormant follicles which have released their fibres.

except during the first winter (age 240-300 days) when they were very similar. The mean skin thickness for the Merino samples was 1.83 mm and for the crossbred

TABLE 3
SUMMARY OF OBSERVATIONS ON FIBRE SHEDDING IN FOUR SOUTHDOWN-MERINO CROSSBREDS

Sheep No.	Age Period Examined	Total No. of <i>P+PS</i> Follicles Examined	<i>Pe+PSe</i> Follicles*		Total No. of <i>S</i> Follicles Examined	<i>Se</i> Follicles*	
			No.	% of Total <i>P+PS</i>		No.	% of Total <i>S</i>
K279	Birth-753 days	982	15	1.53	7,436	67	0.90
K280	Birth-753 days	925	7	0.76	8,538	25	0.29
K283	Birth-752 days	837	6	0.72	6,597	40	0.61
K285	Birth-751 days	1071	5	0.47	8,932	23	0.26
Total		3815	33	0.87	31,502	155	0.49

* Dormant follicles which have released their fibres.

samples was 2.15 mm. There were no marked changes with age, but during the early postnatal period most sheep showed a slight upward trend; other factors appar-

ently influencing skin thickness were nutrition, pregnancy, and shearing. The decrease observed during both autumn–winter periods was probably due to inanition (Wodzicka 1958*b*; Lyne, unpublished observations). This effect, however, was

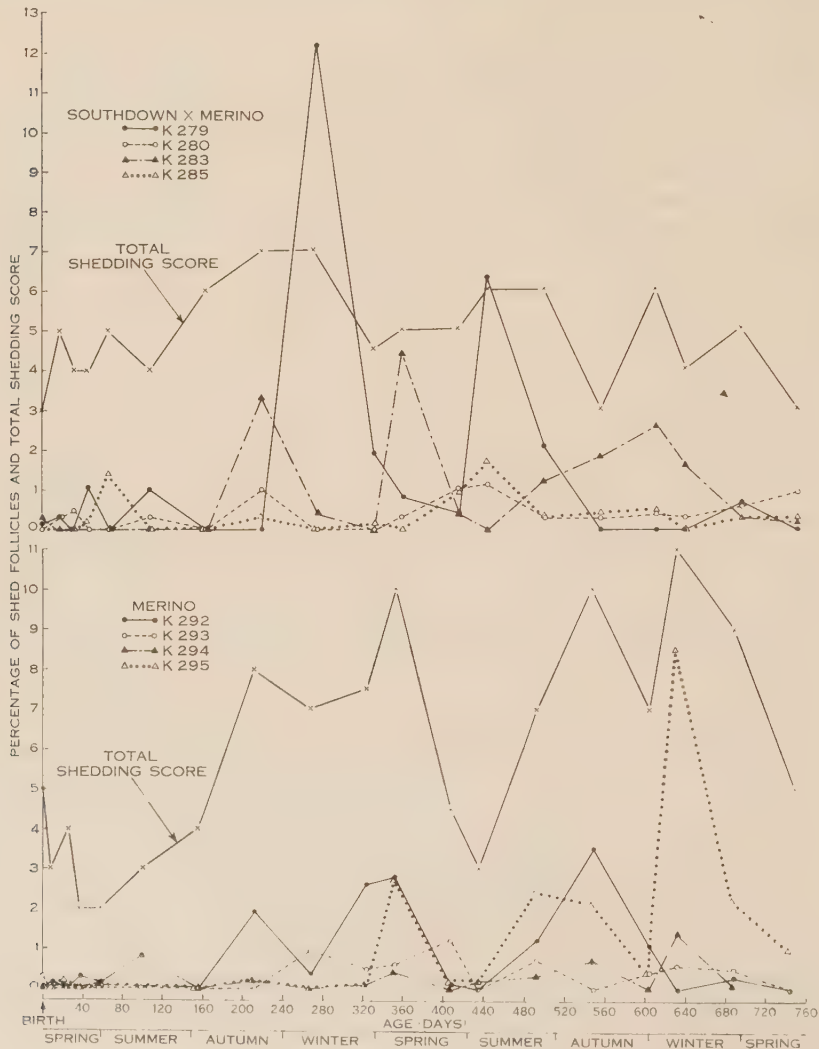


Fig. 9.—Relation between age, season, and percentage of shed follicles in four inbred Merinos and four Southdown–Merinos. The total shedding score (summation of the shedding scores of the four sheep of each group) is also shown.

probably complicated by pregnancy (Fig. 10), which also could have affected skin thickness. The skin increased in thickness immediately after each of the two winter shearings, thus confirming the observations of Wodzicka (1958*c*). The slight increase in thickness after the summer shearing is also in line with observations by Wodzicka-Tomaszewska (1960) that skin increases in thickness as a result of cold stress.

VI. DISCUSSION

(a) *Follicle Population*

A number of authors have made observations on the postnatal development of the follicle population in various breeds. The data on the density of the *P* follicles and fibres are in agreement with the observations of Schinckel (1955*a*), Carter and Tibbits (1959), and others. All the *P* follicles are mature at birth and the decrease in number per unit area during postnatal life is the result of skin expansion.

In observations on the density of mature *S* follicles in South Australian Merinos, Schinckel (1955*a*) describes a "small rise during the first week followed by an extremely large increase in the second and a small rise to a maximum level during the third week. Thereafter, the density falls."

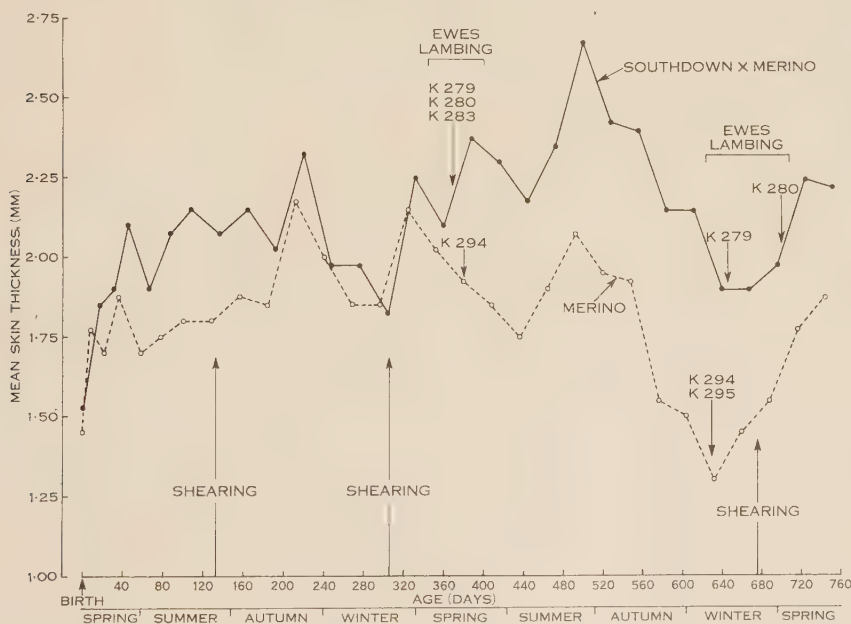


Fig. 10.—Relation between age and mean skin thickness in four inbred Merinos and four Southdown-Merinos.

In the four Merinos examined in the present study the mature *S* density, as well as the mature *P* + *S* density, decreased during the first week after birth. Thereafter, the density rose to a peak at about 25–35 days and then fell rapidly. In contrast to the Merinos, three out of the four crossbreds did not show a fall in the mature *S* density at the second sampling but this was probably due to the fact that these animals were about 9 days older than the Merinos when this sample was taken. One of the crossbreds showed an immediate postnatal fall in the mature *S* density but this was not followed by a rise similar to that seen in the Merinos. As pointed out by Schinckel (1955*a*), changes in the density of the mature *S* follicles are the net result of the interaction of rate of maturation of *S* follicles and the rate of skin expansion accompanying growth. Nutrition has an important influence on the rate

of maturation of the *S* follicle population (Schinckel 1955*b*; Short 1955*a*). Ferguson *et al.* (1956) have also demonstrated the important influence of the thyroid gland.

The follicle population as a whole matured by 110 days in the crossbreds and by 155 days in the inbred Merinos. This confirmed observations on other Merinos and Merino crossbreds (Fraser 1954; Schinckel 1953, 1955*a*; Short 1955*b*).

The increase in the observed number of *S* follicles per *P* follicle in postnatal life in the inbred Merinos is similar to the increase found by Carter and Tibbits (1959) for New Zealand Romney and *N*-type sheep. As pointed out by Carter and Tibbits, apparent delay in *S* follicle initiation may, however, be due to the difficulty of observing all very immature follicles in early postnatal samples. Amongst medium-wool (Peppin) Merinos and Merino crossbreds (Short 1955*b*) and strong-wool (South Australian) Merinos (Schinckel 1955*b*) the *S/P* follicle ratio at birth was greater than the mature *S/P* fibre ratio (up to 6 months of age for the medium-wool Merinos and up to 15 months for the strong-wool Merinos). Both Short and Schinckel claimed that some *S* follicles may not mature if the animal experiences an adverse early postnatal environment.

In various British breeds examined by Burns (1953, 1954, 1955) and Ryder (1957) there was an apparent increase of 1.0 in the *S/P* follicle ratio between birth and maturity. These latter authors claimed that this ratio increase was significant and that it was evidence that follicles were initiated after birth. There does not appear to be any other way of explaining such increases in *S/P* ratio in these British breeds, except that it was not established in the observations of Burns and Ryder that they succeeded in counting all the *Si* follicles in the birth samples; or even that they were aware of the aforementioned difficulties. In Merino and Merino crossbreds most of the immature follicles seen in birth and early postnatal samples are derived secondaries. As these follicles arise by branching, either from the *SO* follicle or from *SD* follicles (Hardy and Lyne 1956*b*), it is virtually impossible to observe all of them at one level in the skin. Consequently, it is important to use sections at the optimal level for counting. Short (1955*b*) has already drawn attention to the importance of the depth of counting of immature follicles if large discrepancies are to be avoided. The level which shows the maximum number of *Si* follicles must be more critical in Merinos than in all other breeds because of the greater frequency of follicle branching. There is no evidence that counts, even at such an optimal level, will include *all* the *SD* follicles in early postnatal samples, and it is most likely that increases in *S/P* ratio, e.g. the increases observed in the inbred Merinos after birth, may have been due to a low count in the early postnatal samples, even though the level of apparent maximum density was selected.

Although several authors (Burns 1949; Narayan 1960) have referred to occasional follicles similar to the primo-secondaries described here, no quantitative observations on these follicles are available. Both Burns and Narayan classified these follicles as primaries despite their aberrant position. The present study supports the view of Burns that follicles form a continuous series in development, and that the usual criteria for distinction between *P* and *S* follicles cannot always be readily observed. If one considers that only *P* follicles develop sweat glands, then the *PS* follicles must be included with the primaries, and it is clear, for example from the

data given here (Fig. 7), that the $(S+PS)/P$ ratio can be substantially higher than the $S/(P+PS)$ ratio.

(b) *Shedding*

The literature on follicle shedding in sheep is not extensive (see Fraser and Short 1960) and there is apparently no information of a quantitative nature available on this aspect for Merino sheep. In the Merino lamb, Carter (1939) states that the P follicles are responsible for almost the entire population of birthcoat kemps (mother hair) which are gradually shed over a period of several months following birth, and then followed by true wool fibres; though in some individuals, and in some regions of the skin more than others, such birthcoat kemps may be succeeded by a second generation of coarse persistent hairy fibres.

Shedding was estimated in the present investigation from the number of dormant follicles without a fibre, though the number found cannot be related, as yet, to any particular period of time, because it is not known how long a follicle might retain a club hair or remain dormant before growing another fibre. The period of time might well depend upon an interaction of fleece-type and follicle type. Very few regenerating follicles were observed at any age which suggests that follicle regeneration is rapid.

There appeared to be no increase in the number of shed follicles at the time of shedding of the birthcoat fibres (2–3 months of age) as noted in other breeds (Fraser, Ross, and Wright 1954; Ross and Wright 1954); in fact rather the reverse (Fig. 9). The published observations, however, refer only to fibres released from follicles whereas the present observations refer only to dormant follicles without their fibres. The number of shed follicles observed during the apparent phase of birthcoat fibre shedding could be small, or could be related to the method of estimation. For instance, the time of dormancy after shedding of birthcoat fibres could be shorter than after shedding in later life. This would mean that relative to the true frequency of quiescent follicles (with or without club hairs) the number of follicles counted without a fibre would be lower in early than in later life. The presently available information is not sufficient to resolve this situation.

The very low incidence of shed follicles suggests that in both the inbred Merinos and Southdown–Merino crossbreeds, follicles have a very long growing phase, and it is interesting to compare the incidence of shed follicles in these animals with that observed by the same method in some other breeds. In various British breeds (see Fraser and Short 1960) the proportion of shed follicles is higher than in the present material.

Clearly, further investigations of the possible cyclic activity of wool follicles now require a more critical approach, exploiting sequential sampling of both skin and fleece to cover age and seasonal trends, with comparisons of divergent fleece-types and adequate control of nutritional and physiological status.

VII. ACKNOWLEDGMENTS

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MICROSCOPIC ANALYSIS OF FAECES, A TECHNIQUE FOR ASCERTAINING THE DIET OF HERBIVOROUS MAMMALS

By G. M. STORR*

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Summary

A technique is described that is being used in diet studies in kangaroos and wallabies. It is based on the identification of the epidermis of leaves and stems of plants recovered in faeces. Data obtained with it are qualitative and quantitative because (1) there is little or no digestion of epidermis that is encased in cutin, (2) epidermis is usually identifiable to species under low-power microscope, and (3) there is a determinable relation in each species of plant between the surface area and dry weight of its foliage.

I. INTRODUCTION

Two events in 1954 stimulated research into the nutrition of the quokkas (*Setonix brachyurus*) of Rottnest I., W.A. First was the observation of a late-summer population "crash" (Waring 1956); then came the discovery by Moir, Somers, and Waring (1956) of the quokka's ruminant-like digestion. Among other projects, work was begun on the trace-element requirements of the quokkas and on the seasonal fluctuation in their health and condition (for preliminary accounts of this research see, respectively, Barker and Barker (1959) and Shield (1959). Development of both studies required a detailed knowledge of the animals' diet. Considerable information had been obtained by observing the incidence of grazing and browsing injury to plants; but such data were only crudely quantifiable and, moreover, were often unassignable to a precise time—a serious defect in view of the seasonal variation in the studied physiological characters.

The methods currently used in studies of livestock nutrition, including those that depend on indicator materials and faecal nitrogen (reviewed by Brown 1954), seemed unsuitable for our purpose. They measure total herbage intake, rather than the fractions of individual food items, which was needed in the Rottnest studies, where in a small area a number of plants of quite different nutritive value may be eaten. A technique was therefore sought that would yield both quantitative and qualitative data for particular times of the year.

Dr. A. R. Main then made the suggestion that quokka faeces, examined microscopically, might furnish the desired information. To test this, penned quokkas were fed for 5 days on a single (and different) item of natural herbage. Their faeces were collected on the sixth day, pounded up, washed, and temporarily mounted. All seven samples contained considerable amounts of epidermal tissue, which differed quite clearly from species to species. It was later found that epidermis from all perennial and a few annual plants passes undigested through the quokka's tract, and that there is no great difficulty in identifying to species the epidermis from plants growing in a restricted area.

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II. PREPARATION OF PERMANENT SLIDES OF PLANT EPIDERMIS

The first requirement in a diet study using microscopic analysis is a reference collection of epidermis from food plants in the study area.

From large leaves an oblong of about 2 by 1 cm is cut; or if small, the leaves are merely trimmed round their edges. Unless this is done, the two surfaces do not separate in the subsequent treatment, which results in an unnecessarily indistinct picture of epidermal characters. Moreover, in plants with dorsiventral leaves it is necessary to have representative tissue from each surface, for they may be very different. As the epidermis from stems is usually modified (see Plate 1, Figs. 1 and 2), separate samples of these are required too, the stems being chopped into lengths of about 2 cm and split longitudinally.

The material is placed in a 150-ml flask containing 5 ml each of 10% nitric and chromic acid, fitted to a reflux condenser, and boiled until the mesophyll disintegrates and the two integuments of each leaf drift apart. This usually requires about 3 min, but in species with very fibrous leaves the operation may take more than 10 min. If a fume-cupboard is available, condensers may be dispensed with; but as there will be no recovery, more acid must be used initially.

After maceration the contents of the flask are poured into a 250-ml beaker, which is topped up with tap water. When the plant fragments have settled, as much fluid as possible is drained off and the beaker is refilled with water to which is added a few drops of ammonia. The supernatant fluid is again drained off and replaced with fresh water. The plant fragments are then transferred to a centrifuge tube, drained, and dyed with an alcoholic solution of gentian violet.

After standing for at least 30 min the tubes are topped up with 70% alcohol which is changed two or three times with upgraded alcohol until excess dye is removed. Changes are facilitated by centrifuging the tubes for 3 min at 3000 rev/min, by which time solids are sufficiently packed at the bottom for the tubes to be safely inverted for draining.

After a final change in absolute alcohol the contents of the tube are poured into a petri dish, whence epidermis is transferred to a slide with a small camel-hair brush. The epidermis is smoothed by gently stroking with a brush and, after removing excess alcohol with blotting-paper, it is mounted in euparal.

III. IDENTIFICATION OF EPIDERMIS

The characters used in diagnosis may be grouped under one or another of five headings.

(a) *Stain Reaction*

As all preparations of foliage and faeces are stained with gentian violet, differences in stain intensity and colour are often helpful. In many species only the vertical walls of cells are stained and the preparation has the appearance of lace (see Plate 1, Figs. 3 and 4). However, in most sclerophylls all parts of the epidermis are evenly and heavily stained. Epidermal tissues usually stain some shade or other of violet, but in a few species the colour is so altered as to be of diagnostic value, and in still fewer species the epidermis is completely unstained.

(b) Orientation

Monocotyledons, especially, are characterized by a definite orientation of cells; in all species examined the ordinary epidermal cells are arranged in rows parallel to the venation and axis of the leaf. This has been observed only rarely in dicotyledons.

(c) Shape, Size, and Inclusions of Ordinary Cells

The unspecialized epidermal cells may be described with respect to their shape, size, walls, and inclusions. Those of dicotyledons are typically hexagonal with a lesser or greater degree of rounding and elongation; in monocotyledons they are elongate rectangular. Within a leaf there is considerable range in cell size, the diameter of the largest commonly exceeding twice that of the smallest. However, the difference in mean cell size between two related species may be so marked as to be diagnostic. Width of cell walls varies from more than 10 to less than 1 μ . Walls may be straight, slightly or considerably curved, undulate, or, as in grasses and sedges, obtusely or acutely crenulate.

(d) Stomata

These may be characterized by the presence or absence of subsidiary cells, orientation of the pore, location, and density. Metcalfe and Chalk (1950) classify the stomata of dicotyledons according to the number and arrangement of subsidiary cells. Three of their five categories are commonly encountered in Australian plants, viz. (1) *anomocytic*, where there are no subsidiary cells, i.e. guard cells are immediately surrounded by undifferentiated epidermal cells; (2) *paracytic*, where there are two subsidiary cells similar in size and shape and whose common wall is co-linear with the aperture; (3) *anisocytic*, where the subsidiary cells (usually three) are of indeterminate size and shape and whose common walls have no fixed relation to the stoma.

Within a species, stomatal cells are generally much more constant in size and shape than are ordinary epidermal cells. Since subsidiary cells may be obscure or absent, it is best for interspecific comparison to consider the size and shape of the area occupied by the two guard cells.

The location of stomata is indefinite in dicotyledons; they occur, if at all, haphazardly throughout the epidermis. The stomata of monocotyledons are usually restricted to definite sites—in most species to between the ends of ordinary cells, but occasionally to rows consisting exclusively of stomata.

In many species of shrubs stomata are completely absent from the dorsal surface of leaves. Elsewhere their density may be as low as 3 and as high as 450 per sq. mm.

(e) Trichomes

The term trichome is a general one for all outgrowths of the epidermis. They are, however, frequently deciduous and so are generally less useful than stomata in diagnosis.

Clothing trichomes usually take the form of hairs, but in succulents they are commonly vesicular, and in a few species they are merely papillose projections from the epidermis. Hairs usually occur singly, but in certain genera and families they are clustered radially about a multicellular base or column. Single hairs may be 1-, 2-,

or 3-cellular. In shape they are typically long, narrow, distally pointed, and proximally wide and flat; exceptional are the short thorn-like hairs of *Spinifex*, the distally rounded hairs of *Frankenia*, the proximally pointed hairs of *Guichenotia*, and the branching hairs of *Solanum*. Vesicles may be sessile or attached to a short, unicellular stalk or a multicellular base. Density of trichomes ranges from 2 per sq. mm in species that are almost glabrous to 700 in species whose ventral leaf surface is completely obscured by hairs.

IV. PREPARATION AND ANALYSIS OF SLIDES OF FAECAL RESIDUES

With a few modifications the preparation of faecal samples follows the procedure, described earlier, for plant material. Faeces should be first dried and thoroughly ground. Owing to its previous digestion the sample need only be boiled in the acid mixture for a minute. Since faecal residues adsorb more dye, one or two additional changes of alcohol are required for the removal of the excess. Furthermore, before changing alcohol it is advisable to centrifuge for at least 5 min lest light material be lost when tubes are inverted for draining.

Slides are examined under low power ($45\times$) by systematically traversing zones 2.8 mm wide (i.e. the diameter of the field of view), whose centres are 5 mm apart. Fragments of epidermis are identified and their area is estimated in hundredths of a square millimetre by using a graduated eyepiece (an apparent millimetre divided into tenths and hundredths). The percentage by area of each species present is then obtained for each slide.

Relative area of epidermis is not in itself meaningful; what is wanted for further analysis is the relative weight of foliage of each species ingested. The relation between dry weight and surface area of leaf varies from species to species; nor is it constant throughout the life of a leaf, as will be seen in the equation

$$W/A = vd/A = td,$$

where W is the dry weight, A the area, v the volume, d the density, and t the thickness of a leaf. However, intraspecific variation in the coefficient W/A is small as compared with that possible between different species; consequently if it is ignored, certain categories of plants will be grossly overestimated in the analysis. A mean value of W/A for each species may be obtained by dividing the total dry weight (in mg) of a representative shoot by its total surface area (in sq. mm). By applying the appropriate coefficients, areas of epidermis are converted into weight equivalent of foliage, and the proportion of each item in a sample is recalculated on a dry weight basis.

Whether data derived in this manner accurately reflect the proportion by weight of the various species ingested is not yet known. At present two assumptions are made: (1) that digestibility of epidermis is approximately the same in all perennial plants; and (2) that sampling errors can be nullified by replication and pooling of data.

V. DIGESTIBILITY OF EPIDERMIS

The assumption that there is no differential digestibility of epidermis among perennial species is based on the more general belief that digestibility of epidermis is all or nothing, the evidence for which is now discussed.

In the preparation of epidermis from annual plants, the foliage of most species disintegrates as soon as the acid mixture reaches boiling point; usually the only part of the epidermis that is recovered is the cuticle. Similar fragments of cuticle are recovered in faeces (where they are measured and recorded as "annual herbs"). On the other hand, the entire epidermis of all perennial species survives maceration and, when eaten, likewise survives digestion. Moreover, epidermis in faeces, apart from being fragmented into smaller clusters of cells, has the same appearance as that macerated *in vitro*; i.e. there is no indication of digestion, such as the erosion of cell walls.

The observed difference in digestibility between annuals and perennials is associated with the distribution rather than the thickness of cutin. Examination under polarized light of transverse sections of leaves of *Medicago* and other annuals

TABLE 1
MEAN PROPORTION BY AREA OF EPIDERMIS OF FOUR PLANTS IN FOOD
AND FAECES
Standard deviations shown in parenthesis

Species	Food	Faeces
<i>Acacia</i>	0.246 (0.086)	0.237 (0.090)
<i>Olearia</i>	0.270 (0.100)	0.268 (0.057)
<i>Rhagodia</i>	0.288 (0.084)	0.306 (0.099)
<i>Scaevola</i>	0.196 (0.113)	0.189 (0.072)

reveals that only the outer wall of epidermal cells is covered by a layer of cutin. In the perennials examined, cutin is deposited on all walls of the cells; the cuticle, in effect, extends down between cells and completely surrounds them (see Plate I, Figs. 5 and 6). Thus the epidermis of perennial plants seems to owe its resistance to both acid maceration and quokka digestion to its being completely encased in cutin.

To ensure further that there is no difference between perennials in digestibility of epidermis, the following experiment was made. A food mixture was prepared consisting of dried, finely chopped *Acacia rostellifera*, *Olearia axillaris*, *Rhagodia baccata*, and *Scaevola crassifolia* (250 g each), made cohesive and palatable with wheaten starch (300 g), sugar (200 g), molasses (130 g), sodium chloride (40 g), and casein (30 g). Three separately penned quokkas (adult females) were deprived of food for 2 days and then fed on the mixture for 5 days. On the morning of the sixth day freshly defecated pellets were collected. A sample of the offered food and a sample of the faeces from one quokka were macerated and stained. From each sample 10 temporary mounts were analysed, the material on a slide being returned to the appropriate sample after examination. The results are set out in Table 1.

In view of the large variation from slide to slide, differences between columns are clearly not significant. Indeed, inter-slide variability is so great that the method could only reveal relatively large interspecific differences in digestibility, which is certainly not the case here. Since the four species tested are a small but representative cross section of the local flora (they are respectively a tall shrub with large hard leaves; a medium shrub with small, soft, hirsute leaves; a scrambling shrub with small succulent leaves; and a low shrub with large subsucculent leaves) the results are consistent with the belief that there is little or no difference in digestibility of epidermis among local perennial plants.

VI. SAMPLING ERRORS

For various reasons the author has always left intact that portion of a faecal sample not immediately prepared into slides. That is, instead of grinding and mixing the whole of a sample and taking from it a convenient-sized subsample, the basic unit for preparation has been the faecal pellet (c. 2 for bettongs, 1 for quokkas and other wallabies, $\frac{1}{2}$ for kangaroos). Since the quokka defecates in the order of 20 pellets per day, our subsample would at first sight seem adequate for determining the diet during a night's feeding. It would be so, if the contents of the stomach were well mixed during digestion; but examination *in situ* of dyed foods shows that the passage of particles through the pylorus is roughly in the sequence of ingestion. Therefore at best, examination of a single pellet can only give information about the diet of an hour or two.

Information for a longer period may be obtained by analysing a subsample drawn from a homogeneous mixture of the whole sample; or, as is the author's practise, by pooling the data derived from analysis of at least 20 pellets, each from a different animal. The latter course is preferable in a population study, since it takes some account of individual variation in food preferences, and gives better coverage of a habitat. Even when the data are finally pooled, it is advisable to analyse separately the faeces of each animal. In this way we have, if required, some information on the diet of individuals; but more important, such data can be reorganized to answer various questions that may arise during the study, e.g. are there differences in diet due to age or sex?

From each quokka pellet two slides are prepared and examined. While usually the same plants are present in each, their proportions are sometimes very different. Discrepancies arise, not from failure to mix adequately the preparation before mounting, but from the great variation in size of epidermal fragments. The area of more than 90% of the fragments is less than 0.3 sq. mm, but occasionally much larger pieces are encountered. The frequency of the latter is so low that their number on any one slide is largely a matter of chance; yet by virtue of their size they may contribute more to the estimate than all other fragments together. The question, then, is how many slides must be pooled before the larger fragments of epidermis no longer greatly disturb mean proportions.

From the results of the feeding trial we have the standard deviations of the proportions of four species of plants in single slides of prepared faeces. These values may be pooled after expressing them as percentages of the appropriate means. Thus

the average standard deviation of data obtained from single slides is estimated as 32%. With this we may calculate the expected standard deviation of means of various numbers of slides from the formula $S_n = S_1/\sqrt{n}$. Expected percentage standard deviations of means of 2, 3, 4, 5, and 10 slides are 23, 19, 16, 14, and 10 respectively. In other words, the probability of an estimate based on the mean of 10 slides being within 10% of the true (or sample) proportion is approximately 67%; and within 20% of the true value, approximately 95%.

Analysis of a large number of slides prepared from a single sample of faeces will only be necessary when precise information is required about the diet of individual animals. More often information will be required about a population. As stated earlier, in population studies the author prepares only one faecal pellet from each quokka, the composition of the preparation being based on the mean of the proportions observed in two slides. Data on the diet of single animals will therefore have an expected standard deviation of 23%. Using again the formula $S_n = S_1/\sqrt{n}$ we calculate the expected standard deviation of means of 10 animals as 7% and of 20 animals as 5%. These estimates necessarily ignore differences between animals to food preferences and differences throughout the study area as to availability of food plants.

VII. DISCUSSION

Although this technique was devised to meet a particular situation, it could well be applied to a wide range of studies. Its special advantage is that the animal may be returned to the study area after a sample of its faeces has been collected. A technique not necessitating the death of the animal is desirable where there are concurrent population studies and is obligatory in research on livestock and on rare and protected animals, as in sanctuaries. The technique could also be valuable in subjects that are hard to trap or shoot but whose faeces are identifiable and easily procured. Even if the animal is shot it makes no difference, at least in macropods, whether material is collected from the stomach or rectum; the contents of all stomachs examined by the author were too thoroughly masticated for macroscopic determination of items.

The chief disadvantage of the technique is its inability to cope satisfactorily with the more tender annual plants, mostly introduced. This seldom matters in macropod studies; for over a large part of Australia this category of plant is absent or ephemeral. In other areas, such as the Wheat Belt or on Rottnest I., these plants may bulk considerably in the diet during winter and spring; but in late summer when the diet is most critical from a nutritional viewpoint, the category is ordinarily absent. It is only in the cooler and more humid areas of southern Australia, and especially where exotic pastures have been established, that the incidence of tender-leaved herbs will be so high that they cannot be ignored.

VIII. ACKNOWLEDGMENTS

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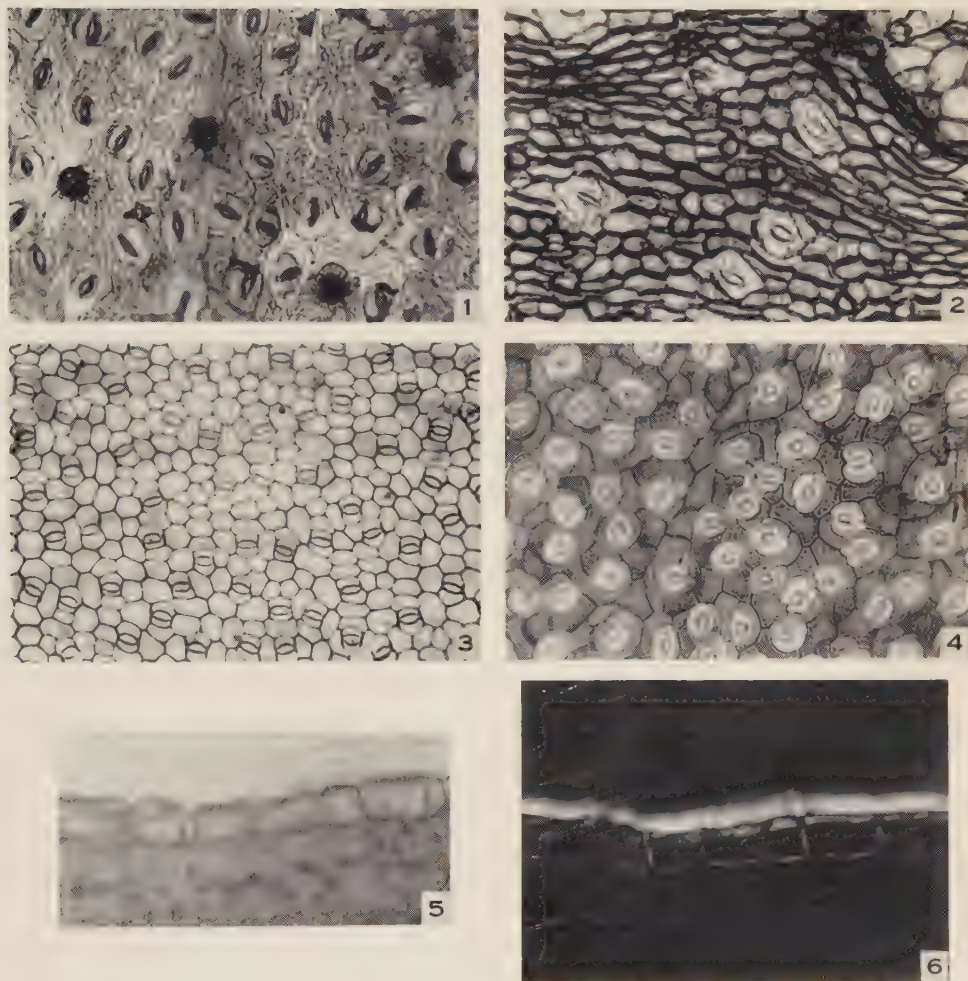
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MICROSCOPIC ANALYSIS OF FAECES



Figs. 1 and 2.—*Myoporum insulare*, showing difference between epidermis from leaf (Fig. 1) and from stem (Fig. 2). In the latter, observe the absence of glands, the elongation of cells, and the thickening of cell walls.

Figs. 3 and 4.—Showing contrast in stain reaction between *Carpobrotus aequilaterus* (Fig. 3) and *Acacia cuneata* (Fig. 4). In the former only the vertical walls of epidermal cells are stained.

Figs. 5 and 6.—Transverse section through epidermis in leaf of *Alyxia buxifolia*, photographed with ordinary light (Fig. 5) and polarized light (Fig. 6).

PATHOLOGY OF INFESTATION OF THE RAT WITH *NIPPOSTRONGYLUS MURIS* (YOKOGAWA)

VI. ABSORPTION IN VIVO FROM THE DISTAL ILEUM

By L. E. A. SYMONS*

[Manuscript received July 19, 1960]

Summary

The net fluxes of water, sodium, and chloride and the absorption of glucose were measured *in vivo* in the distal ileum of rats infested in the jejunum by the nematode *Nippostrongylus muris*. The rate of net fluxes of water and chloride was not affected, but the rate of absorption of glucose and probably sodium was increased in infested rats. The dry weight of the distal ileum per unit length was unchanged by the infestation.

These results are discussed in relation to the previously reported deranged function of the infested jejunum, and it is concluded that malabsorption is not of major importance in nippostrongylosis of rats.

I. INTRODUCTION

In Part III of this series it was reported that the efflux of sodium per gram of dry mucosa from the lumen of the jejunum of rats infested with the nematode *Nippostrongylus muris* (Yokogawa, 1920) was reduced to about one-third of that of normal rats (Symons 1960*b*). The rate of absorption of glucose from the jejunum was similarly depressed. On the other hand, no reduction of the rate of absorption of glucose or of histidine was observed when absorption was measured from the entire small intestine (Symons 1960*c*). It was postulated, therefore, that absorption from the worm-free ileum below the jejunum, the site of the infestation, was not affected by the parasites.

The present paper is an account of measurements in the distal ileum of the net fluxes of water, sodium, and chloride and of the absorption of glucose.

II. METHODS

The preparation of the rats and the method of anaesthetization have been described in Part III (Symons 1960*b*).

(a) Net Fluxes

The *in vivo* technique of Curran and Solomon (1957) as modified by Symons (1960*b*) was used to perfuse loops of the distal ileum of infested and normal rats. The distal cannula was tied into the ileum 3–5 cm proximal to the ileocaecal valve and the proximal cannula about 7 cm above it. The loop was perfused for three periods of 15 min with a solution containing 140 m-equiv. NaCl/l. After perfusion the length of the loop was measured, it was then opened longitudinally and the

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mucus and debris were carefully removed. All the loops from any one experiment were stored at about -10°C until they were dried together at about 100°C .

The net fluxes were expressed as ml or m-equiv/hr/g dry weight tissue. The mean of the three perfusion periods was taken as the rate for each rat.

Sodium concentrations were determined by flame photometry with a Beckman DU spectrophotometer, and chloride concentrations were estimated without protein precipitation, by the method of Schales and Schales (1941).

(b) *Glucose Absorption*

The rate of glucose absorption from the distal ileum was measured by the following methods:

- (1) Nelson-Somogyi: the loops were prepared and perfused as before with a solution of 0.25M D-glucose in Krebs-Henseleit bicarbonate buffer (Umbreit, Burris, and Stauffer 1949). After suitable dilutions of the perfusates, the glucose was determined by the Nelson-Somogyi method (Nelson 1944). This experiment was carried out twice with six normal and six infested rats on each occasion.
- (2) Schaffer-Hartmann: the loops were perfused as before with 0.25M D-glucose in bicarbonate buffer, but the glucose analyses were made by the method of Schaffer and Hartmann (1921).
- (3) One hour perfusion: the loop was prepared as before, except that it was returned to the abdominal cavity and the distal cannula brought to the exterior through a stab wound in the flank. The proximal cannula entered the abdominal cavity through the laparotomy wound which was closed around it. The temperature of the abdomen was raised to 38°C before perfusion was begun with 0.25M D-glucose in 145 m-equiv. NaCl/l. There was no further equilibration period but the rate of perfusion was reduced to 0.1–0.2 ml/min and all the fluid in the collecting tubes and the loop was recovered for glucose estimation by the method of Schaffer and Hartmann. An estimation of water absorption was not possible in this experiment.

Tests for the presence of reducing substances, which might have entered the loop during perfusion, were made by perfusing loops in normal and infested rats with bicarbonate buffer alone. Glucose analyses were made in two separate experiments by the Nelson-Somogyi and Schaffer-Hartmann methods.

The rates of absorption of glucose and water were expressed as mm/hr/g and ml/hr/g of dry ileal tissue respectively.

III. RESULTS

(a) *Dry Weight of Distal Ileum per Centimetre*

The dry weight of the distal ileum per centimetre, measured with the tissue from 12 normal and 15 infested rats, was found to be 0.21 ± 0.003 g in both instances. This result supported the histological evidence reported previously

(Symons 1959) that the infestation did not cause any major anatomical change in the worm-free ileum. It also confirmed the validity of comparing the rates of absorption by normal and infested rats on the basis of the dry weight of the epithelial and muscular layers together.

(b) *Net Fluxes*

The net fluxes of water, sodium, and chloride from the distal ileum of normal and infested rats are set out in Table 1. Nine infested and six normal rats were perfused in this experiment.

In the experiments reported earlier (Symons 1960b), it was found that there was a net influx into the infested jejunum, but in the present experiments there was a net efflux from the ileum of infested rats of sodium, chloride, and water. In

TABLE 1

NET FLUXES FROM DISTAL ILEUM OF NORMAL AND INFESTED RATS PERFUSED IN VIVO WITH SODIUM CHLORIDE

Sodium chloride concentration 140 m-equiv/l. + = net efflux

	Water Flux (ml/hr/g dry tissue)	Sodium Flux (m-equiv/hr/g dry tissue)	Chloride Flux (m-equiv/hr/g dry tissue)
Normal rats	$+7.14 \pm 3.41$	$+1.14 \pm 0.50$	$+2.12 \pm 0.73$
Infested rats	$+4.48 \pm 4.84$	$+1.62 \pm 0.82$	$+2.09 \pm 0.94$
	} n.s.	} $P < 0.05$	} n.s.

a two-tailed *t*-test, the net efflux of sodium was, in fact, significantly greater in the infested rats ($P < 0.05$). There was no statistically significant difference between the net effluxes of chloride and water from the lumen of the ileum of normal and infested rats.

(c) *Absorption of Glucose and Water*

The results of the several experiments to measure the rate of glucose and water absorption from the distal ileum are shown in Table 2, and are set out according to the method of glucose analysis used.

No reducing substances were detected by either method of glucose analysis in the perfusates from the ileum of normal or infested rats perfused with bicarbonate buffer alone. In the first experiment it was found that the distal ileum of infested rats absorbed glucose at a faster rate than did the ileum of the controls, but the difference was small. Furthermore, the results suggested that either the rate of absorption by the normal rats was negligible, or that possibly glucose actually entered the lumen from the tissue during perfusion. For these reasons, the experiment was repeated with the identical technique and with perfusing solutions of the same concentration. In the second experiment, there was a positive absorption of glucose from the ileum of the normal rats, and the rate of absorption was again faster in the infested rats.

Because the large variation of the rate of absorption between individual rats for these two experiments was considered to be due mainly to the Nelson-Somogyi method of glucose determination, the experiment was repeated using the Schaffer-Hartmann method. Again, there was found to be a greater rate of absorption from the ileum of infested rats, and the Schaffer-Hartmann method of glucose analysis reduced the experimental error considerably.

Because the absolute amount of glucose absorbed in one perfusion period of 15 min was small, the experiment was repeated by perfusing for 1 hr and estimating all the glucose in the perfusate and in the loop at the end of this period. By this method it was found again that the infested rats absorbed glucose at a faster rate. The statistical significance of these four experiments is discussed below.

TABLE 2

ABSORPTION OF D-GLUCOSE AND NET FLUXES OF WATER FROM THE DISTAL ILEUM OF NORMAL AND INFESTED RATS PERFUSED IN VIVO WITH D-GLUCOSE

D-Glucose concentration 0.25M. + = net absorption; - = net influx to the lumen

Expt. No.	Methods of Glucose Analysis	No. of Rats Used	Glucose Flux (mm/hr/g dry tissue)	Water Flux (ml/hr/g dry tissue)
1	Nelson-Somogyi	6 normal 6 infested	-0.97 ± 0.98 $+1.06 \pm 1.30$	-3.36 ± 1.49 -2.29 ± 1.38
2	Nelson-Somogyi	6 normal 6 infested	$+2.67 \pm 1.30$ $+4.00 \pm 1.80$	-1.32 ± 0.91 -1.55 ± 1.60
3	Schaffer-Hartmann	7 normal 6 infested	$+0.07 \pm 0.88$ $+0.81 \pm 0.25$	-3.17 ± 3.02 -3.21 ± 2.00
4	Schaffer-Hartmann after 1 hr perfusion	6 normal 6 infested	$+1.83 \pm 0.60$ $+2.84 \pm 1.38$	—

* Analysis of variance: see text for details of statistical analyses.

The net fluxes of water which were measured in the first three of these experiments are also shown in Table 2. In every instance, there was a net influx into the lumen and in no instance was there any statistically significant difference between normal and infested rats.

IV. DISCUSSION

The finding that the dry weight of the ileal tissue per unit length of normal and infested rats was identical contrasts with the dry weight of jejunal tissue which was increased by 50% per centimetre in the infested rats (Symons 1960a). The similarity of the dry weights of the ileum of normal and control rats justified the decision to express the rates of absorption per unit dry weight of entire tissue in the present experiments. It was not necessary to separate the mucosa from the muscle layers as was done in the jejunal experiments.

A comparison can be made between the net fluxes of water and sodium found in the present experiments and those reported by Curran and Solomon (1957) who perfused the distal ileum of rats by the *in vivo* technique upon which these experiments were based. The net fluxes of water and sodium for normal rats in Table 1, when converted by the ratio of dry weight/cm to 0.15 ml and 0.023 m-equiv/hr/cm respectively, are within the upper limits of the wide range shown by Curran and Solomon for perfusion with a solution containing 150 m-equiv. NaCl/l. They did not state a similar range for net chloride fluxes, but approximate net rates derived from other data in their paper suggest that the net fluxes in the present experiment were higher than they obtained. On the other hand, the fluxes presented now tend to support the finding of Curran and Solomon that the net chloride flux is greater than the net sodium flux.

A comparison of the rate shown in Table 2 for glucose absorption from the distal ileum of normal rats with the rate of absorption from the jejunum of normal rats reported earlier (Symons 1960c), suggests that the former is lower. This confirms the reports of *in vitro* experiments with the small intestines of rats by Fisher and Parsons (1950) and hamsters by Korelitz and Frank (1959), who found that the rate of absorption increased with the proximal distance from the ileocaecal valve.

There was no evidence in the present experiments that the net influx of water, sodium, and chloride and the depression of absorption of glucose that occurred in the jejunum of infested rats occurred in the distal ileum. There was, in fact, a strong indication that a compensatory increase in the rate of absorption occurred. The net efflux of chloride from the ileum of infested rats did not differ significantly from that from the ileum of normal rats, but there was evidence of an increase in the rate of net sodium efflux, although slight, and significant only at the 5% level. An increase in the rate of net efflux of sodium is, however, not necessarily evidence of increased rate of absorption. The increase found could have been due to a reduction in the rate of influx into the lumen without any change in the rate of efflux. Only measurement of the unidirectional fluxes could decide this point.

The results of the glucose absorption experiments do, however, indicate a compensating faster rate of absorption from this region of the small intestine. No single experiment provided unequivocal evidence that glucose was absorbed faster from the distal ileum of infested rats, but the fact that in each experiment the rate was greater in these animals strongly suggested that this was so. It was not possible to analyse the four experiments together, but an analysis of variance showed that although the variation between the two Nelson-Somogyi experiments was highly significant ($P < 0.001$), the variation between the normal and infested rats was also significant ($P < 0.01$). This result refuted the original hypothesis that absorption from the distal ileum was not affected by the infestation, and provided evidence that the infestation increased the rate of absorption from this section of the small intestine. One-tailed *t*-tests of the hypothesis that the mean rates of absorption in normal and infested rats were equal, against the alternative hypothesis that the infested rats absorbed at a faster rate, were then made on the two Schaffer Hartmann experiments. These tests were found to be significant at the 5 and 7% levels respectively. In order to summarize the results of the three independent statistical tests

which were made of the data of the four experiments, the exact probabilities associated with each test were calculated and compounded according to Wallis (1942). The resulting compounded probability was less than 0.001. This indicates that there is no reasonable doubt that the infestation increased the rate of absorption of glucose from the distal ileum. The extent of this increase cannot be estimated with any accuracy from the results available, but may be only slight.

The net movement of water across the epithelium of the distal ileum was measured under two conditions. From a solution containing 140 m-equiv. NaCl/l only, there was a net efflux of water from both normal and infested rats, the rates of which were not statistically significantly different, although the mean rate from the infested ilea was lower. In the glucose-absorption experiments the solutions were hypertonic because the bicarbonate buffer in the first three, and the sodium chloride in the last experiment were not adjusted osmotically to allow for the added glucose. This accounts for the net influx which occurred in all instances, and the results accord with the findings of Curran and Solomon (1957). Symons (1960c) showed that hypertonic solutions also cause a net influx into the infested jejunum; therefore, the present experiment does not exclude the possibility of the infestation affecting water fluxes in the ileum.

In the earlier experiments, an apparent contradiction was found between the deranged function of the infested jejunum and the unaffected rate of absorption of glucose and histidine from the small intestine as a whole (Symons 1960b, 1960c). This may now be explained by the compensating increase in the rate of absorption of glucose from the ileum. This explanation is supported by the probable increase in the rate of absorption of sodium.

Alternatively, however, it is now possible to envisage normal physiological responses which would ensure that the products of digestion, which are not absorbed by the infested jejunum, would be absorbed by the unaffected ileum. Borgström *et al.* (1957) have shown that all fat and carbohydrate and the greater part of protein fed to rats was absorbed before the ingesta reached the lower ileum, chiefly from the duodenum and jejunum. Schlüssel and Sunder-Plassmann (1953) also found that the greater part of protein absorption occurred in the first half of the small intestine. Reynell and Spray (1956) have shown that as much as the upper two-thirds of the small intestine of the rat may be resected without loss of weight or demonstrable defects of absorption. These findings indicate a large functional reserve in the small intestine. Furthermore, several workers have shown that, within limits, there is a direct, though not necessarily linear, relationship between concentration in the lumen and rate of absorption (Groen 1937; Fullerton and Parsons 1956; Acland and Illman 1959; Jervis and Smyth 1959). It can be postulated, therefore, that the products of digestion will reach the ileum of the infested rats at higher concentrations than will occur normally, and that the rate of absorption will be correspondingly faster. It must be emphasized that in the experiments presented in this paper, the normal and infested rats were perfused with similar solutions, so that the increased rate of glucose absorption which is reported is not due to a higher concentration in the lumen of the ileum. It is not known whether the increased rate of absorption due to the infestation will alter the response of the ileum to different concentrations in the lumen.

It was shown in Part V of this series (Symons 1960*d*) that the digestion of protein was considerably depressed by the infestation and absorption of the products may have been slightly reduced. Because it can now be stated that the absorption of carbohydrate at least is not affected, it is concluded that a relative failure of digestion rather than malabsorption of its products is the more important aspect of nippostrongylosis of the rat.

V. ACKNOWLEDGMENTS

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STUDIES OF DORMANCY IN THE SEEDS OF SUBTERRANEAN CLOVER (*TRIFOLIUM SUBTERRANEUM* L.)

II. THE INTERACTION OF TIME, TEMPERATURE, AND CARBON DIOXIDE DURING PASSAGE OUT OF DORMANCY

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Summary

The dormancy-breaking action of CO₂ on imbibed seeds of subterranean clover (*Trifolium subterraneum* L.) is temperature dependent, the efficiency falling off sharply above 25°C and approaching zero at 30°C. Within the effective temperature range the action is rapid, exposure of seeds to CO₂ for 6 hr having significant effect.

Freshly harvested seeds of dormant strains do not germinate at 22°C or higher temperatures; but do so at 11°C and lower temperatures. When held at temperatures between 3 and 11°C for 1 and 3 days, and then transferred to 22°C, the germination of freshly harvested seed is lower than if no transfer had been made; but it is higher the longer the duration of cold treatment. The extent of the high temperature inhibition of germination of both young and old seed depends both on strain and age of seed.

An hypothesis is advanced in which germination is assumed to be dependent on the level of some carboxylated compound. The dormancy-breaking actions of CO₂, of activated carbons, and in part at least of low temperature, are interpreted in terms of net carboxylation.

In addition to this control mechanism, evidence is presented for the existence of at least two other steps in the germination sequence. The first is the passage out of a stage of "ultra-dormancy", during which seeds are sensitive neither to low temperature nor CO₂ treatment. Ultra-dormancy has been noted only in the most dormant strains, and only in some seasons. The second is the formation of a precursor of the carboxylated compound.

I. INTRODUCTION

In Part I of this series, the dormancy-breaking action of CO₂ on the seeds of subterranean clover (*Trifolium subterraneum* L.) was first described (Ballard 1958), and G. B. Lipp and Ballard (1959) have concluded that this effect is general among small-seeded legumes.

The purposes of the present paper are (1) to present data further characterizing the CO₂ effect, mainly with respect to interactions with time and temperature, and on the basis of these and other facts (2) to present an hypothesis for the mechanism of CO₂ action.

* Division of Plant Industry, C.S.I.R.O., Canberra.

In describing results of germination tests it is common practice to refer to rate of germination, and measures have been devised to express this quantitatively. Either the time for a sample to reach any arbitrary level of germination, or indices such as those developed by Bartlett (1937) and Durand (1953) have been used.

These measures, however, refer to the sample, and yield relatively little information about an individual seed. Moreover, since some criterion such as the emergence of the radicle or its positive geotropism must necessarily be adopted, germination, as usually measured, is an all-or-none process. But most probably many steps or reactions are involved. Although these may form a continuous sequence it is useful to distinguish between those at the beginning of the sequence, which are concerned with "activation" of the dormant embryo, and those at the end which are mainly concerned with extension growth (Toole *et al.* 1956). Thus any estimates of rate of germination, even of an individual, may be the resultant of differing rates of, e.g. the "activation" and "growth" phases. Considerations of this nature are relevant in attempting to interpret rates of germination in physiological terms.

For subterranean clover it is known that both freshly harvested (young) and stored (old) seeds held at high temperatures germinate to a low level and at a low sample rate (Toole and Hollowell 1939; Loftus Hills 1944*a*). Young seeds held at low temperatures (Woodforde 1935; Loftus Hills 1944*a*; Ballard 1958) as well as in atmospheres of low CO₂ tension (Ballard 1958) germinate to a high level and with a high sample rate. Analyses of the processes involved have not so far been attempted.

II. METHODS

Procedures were essentially as described in Part I (Ballard 1958). All experiments were carried out with imbibed seeds, which, except for the special cases referred to in the text, were held at 22°C. Temperatures higher than 10°C were obtained with an accuracy of at least $\pm 1^\circ\text{C}$, those lower than 10°C with considerably less accuracy. These conditions are therefore referred to as temperature ranges, the means of which were clearly separated, although some overlap at the extremes occasionally occurred.

Carbon dioxide treatments were effected by the displacement method. The concentration employed, unless otherwise stated, was 2.5% by volume; that and other figures quoted referring to initial concentration (see comment concerning drifts in Part I).

Since, for the analysis attempted, an appreciation of the evident broad trends is sufficient, most of the data are presented graphically. Each point of a graph represents the mean of a number (usually 4) of replicates of 50 seeds (25 seeds for activated carbon and CO₂ treatments). The angular transformation of percentage figures was used where analyses of variance were carried out.

III. RESULTS AND INTERPRETATION

(a) *Temperature Relationships*

(i) *Old Seeds Held at Constant Temperatures.*—On the basis of rate of germination at 22°C (Loftus Hills 1944*a*, 1944*c*) the strain Tallarook shows little or no dormancy

immediately after harvest, and the strains Mt. Barker and Burnerang are moderately and strongly dormant respectively.

Seeds of the above three strains which had been stored in the laboratory for approximately 17 months after harvest were then imbibed and held at 3–5, 10, 15, 22, 25, and 30°C. The time course of germination for each strain is given in Figure 1. The values at 25°C are not plotted for Tallarook and Mt. Barker since they are almost exactly the same as those at 22°C.

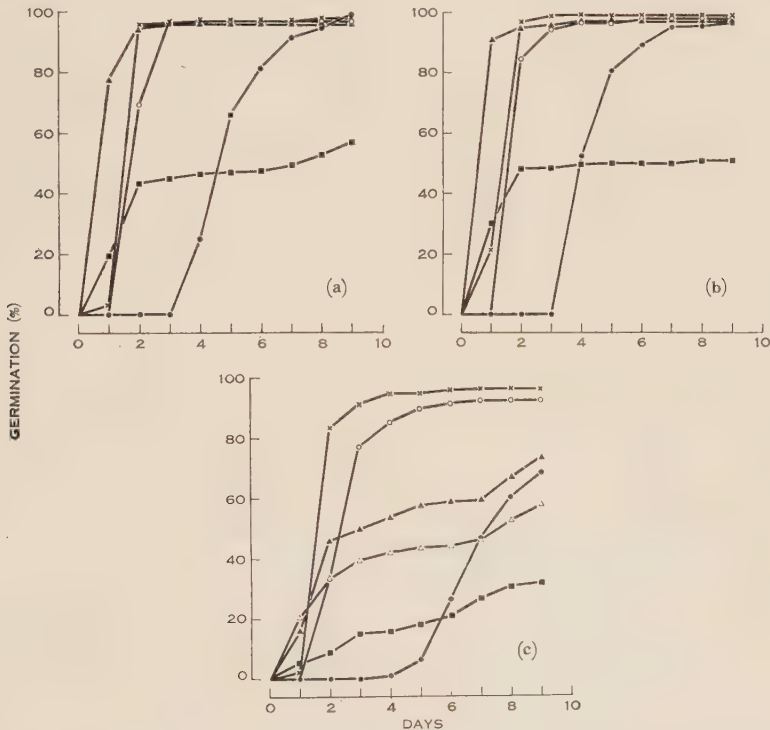


Fig. 1.—Time course of germination for three strains of subterranean clover, Tallarook (a), Mt. Barker (b), and Burnerang (c) at various temperatures, approximately 17 months after harvest. ● 3–5°C; ○ 10°C; × 15°C; ▲ 22°C; △ 25°C; ■ 30°C.

For Tallarook and Mt. Barker (which were no longer dormant) the broad picture of an optimum sample rate of germination close to 22°C agrees with earlier findings, even though Loftus Hills (1944a) averaged his results over five strains. The strain used by Toole and Hollowell (1939) was not recorded; in this case the decline from the optimum is much more marked than in the other two sets of data.

For both Tallarook and Mt. Barker, full germination ensued not only at 25°C but also at all lower temperatures. Except for the increasing delay in the onset of germination with decreasing temperature, the forms of the germination curves are similar. The delay is probably to be attributed in greater part to the effect of temperature on the growth phase.

On the other hand storage for 17 months was insufficient to effect full release from dormancy in Burnerang, and full germination occurred only at 15°C and lower

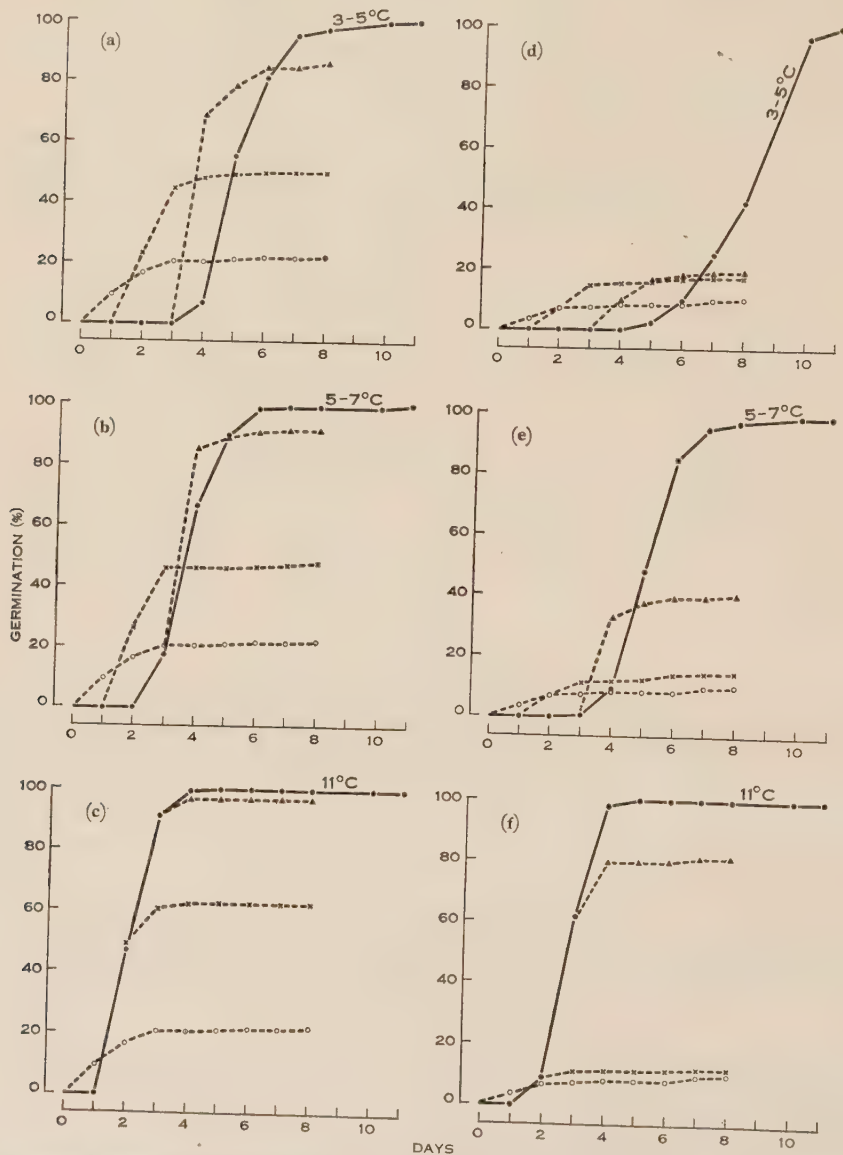


Fig. 2.—Effect of three levels and two durations of prechilling on germination of two strains of subterranean clover. (a), (b), and (c), Mt. Barker; (d), (e), and (f), Burnerang, 7 and 6 weeks, respectively, after harvest. ● Permanently at low temperatures; ○ permanently at 22°C; × transfer to 22°C after 1 day low temperature; ▲ transfer to 22°C after 3 days low temperature.

temperatures. At 22, 25, and 30°C increasingly marked inhibition of germination occurred.

Among the three strains, and for the several different temperatures at which inhibition occurred, the forms of the germination curves are generally similar, and dissimilar to those showing complete germination.

(ii) *Young Seeds and Pre-chilling*.—Seeds of the strains Mt. Barker and Burnerang which had been stored in the laboratory for 7 and 6 weeks respectively after harvest were then imbibed and held at 3–5, 5–7, and 11°C for 1 and 3 days, after which they were transferred to 22°C. Other lots were held continuously at each of the three low temperature ranges, and at 22°C. The time course of germination in each case is presented in Figure 2. Both strains give an entirely consistent and qualitatively similar picture, the details of which are more readily seen in the Mt. Barker curves.

The significant features are: (1) the close similarity of those lots held permanently at low temperatures, with those of the corresponding temperature for *old* seed (Fig. 1); (2) the similarity of the 22°C lots with the 30°C lots of *old* seed; (3) after transfer from a low temperature to 22°C the asymptotic level is lower than if no transfer had been made; (4) this asymptotic level is higher the longer the duration of low temperature.

TABLE 1
GERMINATION RESPONSE TO CARBON DIOXIDE AT 30°C

Values are mean cumulative percentage germinations at day 7. Burnerang strain

Initial CO ₂ concn. (%)	Atmospheric*	1.3	2.5	5	10	20
Germination (%)	14.8	24.0	28.0	28.0	26.5	29.0
	Group A	Group B†				

* Not sealed.

† No significant differences between members of group B. Group B significantly different from group A at $P < 0.01$.

Further, neither very low temperatures, nor long exposures to them, are necessary for dormancy breaking by cold. The best of the three temperatures investigated was 11°C (and the optimum therefore lies between 7 and 22°C). In both strains full germination was reached in 4 days at this temperature.

(iii) *Effect of Temperature on Carbon Dioxide Response*.—The evidence already presented (Ballard 1958) (as well as further examples found in Figures 3 and 4) refers to the action of CO₂ at 22°C. Evidence on the efficacy at higher temperatures is desirable because of their adverse effects noted above.

The data of Table 1 show that at 30°C no treatment differences exist over the range 1.3–20% initial CO₂. While the CO₂ treatments as a whole show a statistically significant promotion of germination compared with 30°C controls, the absolute germination is small compared with that at lower temperatures. The data of Figures 3(f) and 5 refer to the same sample, serving as controls for its capacity for

promotion. The data of Figure 3(f) were obtained 3 weeks earlier (and hence under greater dormancy) with 2.5% CO₂ at 22°C and those of Figure 5 simultaneously

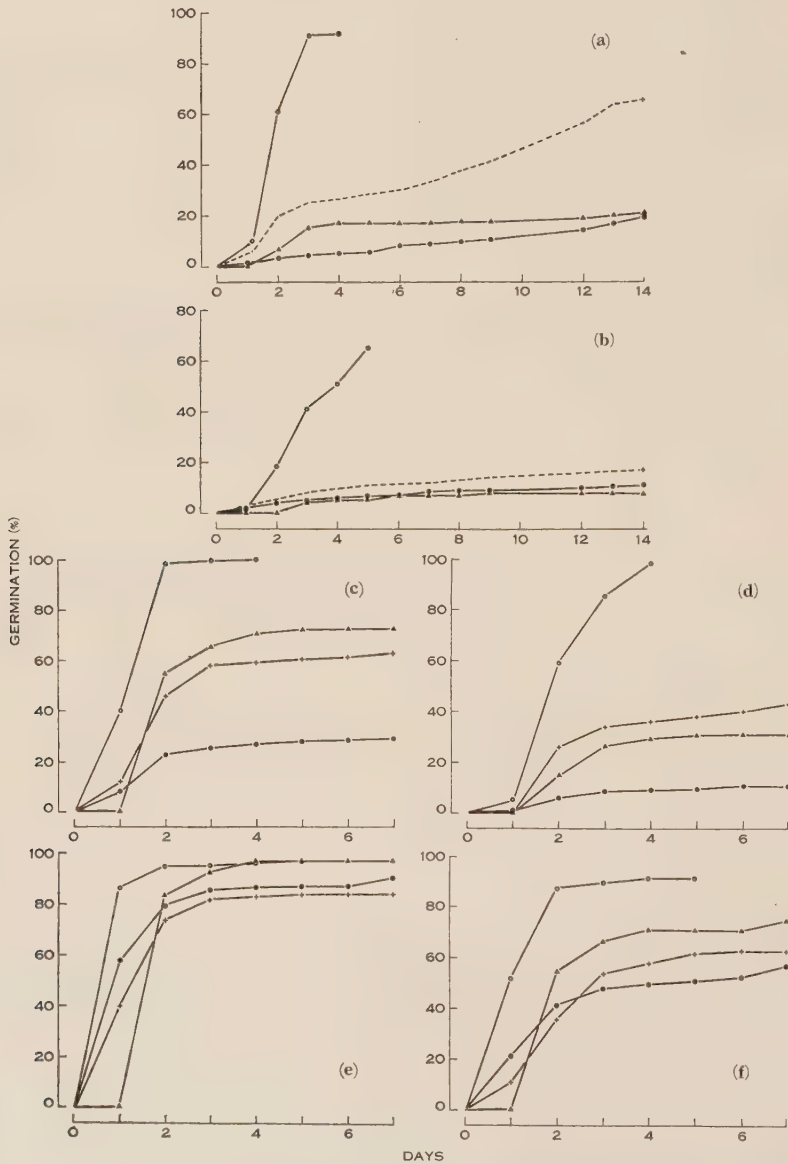


Fig. 3.—Time course of germination for two strains of subterranean clover at various intervals after harvest, and under three dormancy-breaking treatments. (a), (c), and (e), Mt. Barker, 2, 9, and 18 weeks, respectively, after harvest; (b), (d), and (f), Burnerang, 1, 8, and 17 weeks, respectively, after harvest. ● Control; ○ 2.5% CO₂; + activated carbon; ▲ 1 day at 3–5°C.

with the data of Table 1, with 2.5% CO₂ at 25°C. In both these latter cases full promotion occurred.

Full germination also ensued in the single trial where CO_2 was applied at low temperature (10°C), and the sample rate of germination was greater than in a 10°C treatment alone, or CO_2 at 25°C .

The summary conclusion is that CO_2 treatment is effective at temperatures up to 25°C —beyond this the efficacy declines sharply, and the treatment is unable completely to relieve the high temperature inhibition.

(b) *Time Relationships*

(i) *First Category: Early Response to Carbon Dioxide.*—In order to ascertain whether the dormancy-breaking action of CO_2 is related to the post-harvest age of seeds, lots of the strains Mt. Barker and Burnerang were subjected to standard CO_2 promotion conditions after being stored for three different periods. Comparison was made with control lots, with lots held one day at $3\text{--}5^\circ\text{C}$ prior to transfer to 22°C , and with lots treated with activated carbon (Ballard 1958). The results are given in Figure 3.*

The most conspicuous feature of the curves is the response to CO_2 . In Mt. Barker virtually complete germination ensued in all cases irrespective of the control germination. The sample rate of the CO_2 -induced germination was greater, the greater the control germination (i.e. the less the dormancy). Essentially the same picture is given for the more dormant Burnerang. Unfortunately, observations were not continued beyond day 5 at the first test, so it is unknown whether full germination could be reached here also. Other evidence suggests that it could.

Earlier it was noted that, among several strains showing a range of dormancies when tested at the one time, there appeared a tendency for activated carbon to be a more effective treatment than low temperature in the more dormant cases, and the reverse in the less dormant cases. The same tendency is to be seen in this set of data, where the range of dormancies arises, in time, in single strains. No explanation can yet be offered.

The above data were obtained using seeds produced in 1955, and a similar behaviour especially with respect to the response to CO_2 shortly after harvest, has been observed in other seasons.

(ii) *Second Category: Delayed Response to Carbon Dioxide.*—When seeds produced during 1956 were tested under the same general conditions, a different type of response was observed among extremely dormant strains. The strains Mt. Barker, Burnerang, and Portugal C.P.I. 19465† were tested 7, 7, and 4 weeks, respectively, after harvest, and again at 13, 13, and 10 weeks after harvest. Carbon dioxide (initial 2.5%) and low temperature (1 day at $3\text{--}5^\circ\text{C}$) treatments were applied.

* It was necessary to use two samples of activated carbon with slightly different characteristics—one for the first storage point, and the other for the two later storage points. On the basis of a comparison carried out at the time of the second storage point, the actual values obtained with the first carbon sample were adjusted to correspond to the activity of the second carbon sample, thus giving the dotted lines of Figures 3(a) and 3(b). It is recognized that this is an approximation, since the relation between the activities of the two carbon samples may differ on seeds of different post-harvest ages.

† Commonwealth Plant Introduction number.

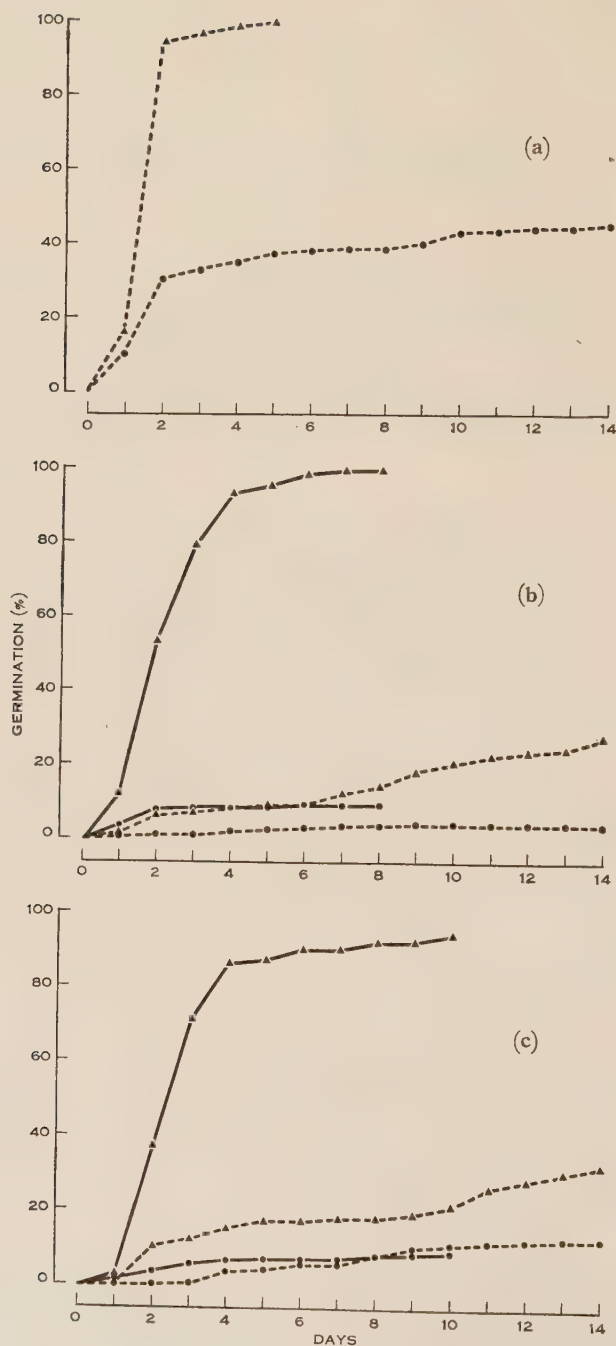


Fig. 4.—Effect of length of storage on sensitivity to CO₂ for Mt. Barker (a), Burnerang (b), and Portugal 19465 (c) strains of subterranean clover. ● Control; ▲ 2.5% CO₂. ---- 7, 7, and 4 weeks storage, respectively, for Mt. Barker, Burnerang, and Portugal 19465 strains. ——— 13 and 10 weeks storage, respectively, for Burnerang and Portugal 19465 strains.

The results for the CO_2 treatments are presented in Figure 4. Whereas the behaviour of Mt. Barker follows exactly that depicted in Figure 3, CO_2 elicited only a slight germination in Burnerang at the first test, in contrast to the behaviour seen in Figure 3(b), although this latter test was conducted 6 weeks earlier in storage life (and hence potentially at a more dormant stage). The response of Portugal 19465 was similar to that of Burnerang. Six weeks later, when the control germination of Burnerang had increased only very slightly, and that of Portugal 19465 not at all, CO_2 treatment elicited full germination.

The results of the low temperature treatments, not presented in detail, were as follows. For all strains, whenever CO_2 was ineffective, so also was low temperature. Whenever CO_2 was effective, low temperature also increased germination, but less markedly and to levels generally comparable with those of Figure 3.

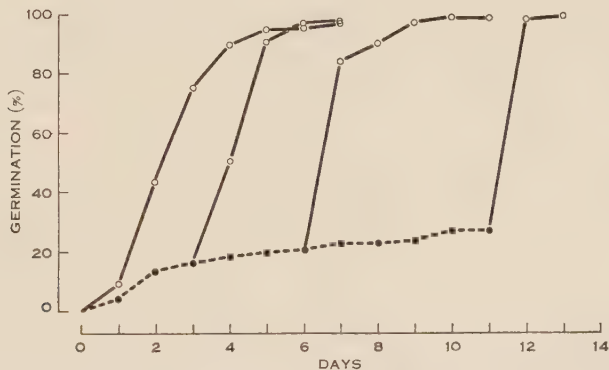


Fig. 5.—Effect of duration of imbibition time on sensitivity to CO_2 at 25°C in the Burnerang strain of subterranean clover. ● Without CO_2 ; ○ after transfer to 2.5% CO_2 .

The essential feature here is the marked change in responsiveness to CO_2 during a period in which no, or only a very slight, change in control germination occurred. Grant Lipp and Ballard (1959) observed a similar behaviour in the dormant seed of the legumes *Trigonella ornithopodoides*, *Trifolium cherleri*, and *Trifolium hirtum*, indicating that the present result with the highly dormant subterranean clover strains is not an isolated experience.

(iii) *Effect of Length of Imbibition Period.*—In earlier experiments it appeared that the rate of germination resulting from dormancy-breaking treatments was greater if the seeds had been held imbibed for some period prior to the application of the treatment. Evidence to this effect is now presented for the case of CO_2 treatment.

These experiments were conducted as follows: Some thousands of seeds were set out in large dishes and observed daily. Germinated seeds were counted and discarded. At intervals, randomly selected replicates of the remaining ungerminated seeds were transferred to bottles containing 2.5% CO_2 at the time of sealing.

From the known initial number of seeds, the number of germinated seeds removed, the number of seeds transferred at intervals to CO₂ treatments, and daily germination counts of the latter, time course curves of percentage germination were constructed.

In one experiment, the results of which are given in Figure 5, seeds received CO₂ treatment commencing 0, 3, 6, and 11 days after imbibition. At 25°C,* while the control sample reached some 27% germination, CO₂ promoted virtually complete germination, though somewhat more slowly than at 22°C.

TABLE 2

EFFECT ON GERMINATION OF DURATION OF CONTACT OF CARBON DIOXIDE WITH SEEDS

Values are mean cumulative percentage germinations. Pre-incubation period 8 days. CO₂ concn., 2.5%; temperature, 25°C. Burnerang strain

Treatment	Germination (%)		Treatment	Germination (%)	
	Day 1	Day 5		Day 1	Day 5
Permanently open	11.3	17.3	6 hr CO ₂	61.0	61.0
Permanently closed	60.0	100	6 hr closed	15.0	17.0
Permanent CO ₂	98.0	100	12 hr CO ₂	92.0	92.0
			12 hr closed	22.0	23.0

Any appreciation of the rates of germination among the various CO₂-time treatments is rendered difficult by the fact that the CO₂-elicited germination is superimposed at different points along the rising control curve. Ideally, a comparison of the rates following CO₂ treatment might have been made by transformation of the percentage germination data to give time-linear curves, and then comparing their slopes. Insufficient points, particularly in the longer imbibition treatments, are available for this. An approximation, which increasingly *underestimates* rates the longer the imbibition period, is to estimate the rates of germination at the time 50% of seeds have germinated, assuming a linear rate between the two daily values which are respectively next lower and higher than 50%. In this experiment, this measure, expressed as percentage germination per day, is 32.0, 37.4, 63.5, and 71.7 for CO₂ treatments commencing respectively 0, 3, 6, and 11 days after imbibition, i.e. the rate increases with increase of the imbibition period prior to CO₂ application.

(iv) *Required Time of Contact with Carbon Dioxide*.—In Part I it was shown that contact of seeds with activated carbon for as little as 4 hr was sufficient to produce a significant increase in germination. Since it is suggested that activated carbons produce their effect via CO₂, it would be expected that similar brief exposure to CO₂ should also be effective.

* Experiments described in this and Section III(b)(iv) were carried out on seeds with reduced dormancy after storage for 5-7 months. Under these conditions any possible differences between control and treatment values are minimized. Greater discrimination was obtained by the use of a temperature of 25°C instead of 22°C elsewhere employed.

Experiments to investigate this feature were carried out in germination bottles containing 2.5% CO₂ at the time of sealing. After the lapse of the required time, the CO₂ was removed by adequate perfusion with air, and the bottles thereafter remained open. Controls consisted of bottles permanently closed containing 2.5% CO₂, permanently open, permanently closed, and closed for the duration of any CO₂ treatment and thereupon opened, perfused with air, and remaining open.

Experiments conducted at 25°C showed no evidence for any effect of CO₂ when it was applied for periods up to 12 hr at the time of imbibition. However, when the CO₂ treatments were applied after a preliminary incubation period, as described in the previous section, significant increases were observed for a contact time of 6 hr. The data of Table 2 refer to the dormant seed (some 83%) remaining after a preliminary incubation period of 8 days.

The somewhat reduced sensitivity compared with that observed for activated carbon, may, in part, be due to the higher temperature—at all events it is clear that under certain circumstances the response to CO₂ is also very rapid.

IV. DISCUSSION

(a) *The Role of Carbon Dioxide*

With few exceptions (Anderson 1933; Thornton 1935, 1936) treatment of seeds with CO₂ has not proved stimulatory of germination; but rather induces secondary dormancy (Kidd 1914*b*; Thornton 1953). Since concentrations of CO₂ as low as 0.5% are effective in inducing germination in subterranean clover seeds, which are well buffered because of their high protein content, it is unlikely that the effect arises simply through a marked shift in pH. This conclusion is supported by the uniformly effective action of both acidic and alkaline active carbons (Ballard 1958).

It seems that some metabolic function should be assigned to CO₂, and the hypothesis is advanced that one of the sequential steps leading to germination of subterranean clover seeds is a carboxylation, and that in the absence of sufficient carboxylated product, C, germination is blocked. Normal passage out of dormancy would then follow the slow accumulation of C, and strain variation may result from differences in the carboxylation reaction such as the time of formation of the enzymes concerned, in relation to time of maturity on the maternal plant.

Many carboxylating systems from plant tissues are known (for review see Vennesland and Conn 1952); but no evidence at all exists as to the detailed nature of any possible system which may be concerned in this germination response. However, the feasibility of the general hypothesis is supported by the existence of other cases where CO₂ controls some developmental process. Mer and Richards (1950) and Mer (1952, 1957) have noted the stimulation of mesocotyl elongation of dark-grown *Avena* seedlings by CO₂. Loomis (1957, 1959) has shown its decisive function in sexual differentiation in *Hydra*, and Trinkaus and Drake (1959) for morphogenesis in *Fundulus*. Cantino (1951, 1956) has shown that morphogenesis in *Blastocladiella* is under the control of a sensitive bicarbonate "trigger mechanism"

and a well-documented account has been provided of the biochemical steps in the reductive carboxylations concerned both in morphogenesis, and in a CO_2 -stimulated growth also observed in this organism (Cantino and Horenstein 1956, 1957, 1959).

(b) The Role of Temperature

The facts presented in Sections III(a)(i) and III(a)(ii) make it clear that high temperatures are inhibitory to germination, and further, that the younger the seed the lower is the inhibitory temperature. In fact it may be possible to define dormancy more quantitatively than at present in terms of the minimum temperature required to produce some prescribed level of germination.

At least two interpretations of the temperature phenomena are possible. It could be assumed that the necessary condition(s) for germination already exist in young seeds, and thus low temperature merely ensures the absence of inhibition. Alternatively, low temperature may positively induce a condition not present in young seeds, and which is necessary for initiating the germination sequence. The data of Figure 2, which show a high Q_{10} for the rate of germination between 3 and 11°C, favour the latter alternative; but are not decisive because of the difficulty of dissociating any (presumed) initial step, and later ones. It is not readily apparent how this could be done, since subterranean clover seeds will eventually germinate at temperatures close to 0°C, but do not, in the imbibed state, survive prolonged exposure to temperatures below 0°C.

(c) Relationships between Germination-inducing Agents

Dormancy may be abolished or reduced by several treatments of intact imbibed seeds, and also by removal of the seed coats. This raises two interrelated questions. Do the various treatments activate common or different mechanisms, and what are the sites of such mechanisms? Only partial answers can yet be given.

For intact seeds two main lines of evidence suggest the action to be on the embryo. Firstly, Morley (1958) has shown that the seed germination of strains, the dormancy of which is known to be relieved by both low temperature and CO_2 treatments, is dependent, at least in part, on the genotype of the embryo. Secondly, it is known that in cases where removal of the testa does not induce germination, treatment of the resulting dormant embryos with CO_2 or activated carbon usually does (Ballard 1958).

Insufficient evidence is available to reach a decision whether low temperature and CO_2 operate on the same or different mechanisms. Stimulation of seed germination by low temperatures is a widely observed phenomenon, and it is possible that this treatment facilitates, in each case, an alternative pathway to germination.

In subterranean clover, and other legumes, it is also possible that low temperature in some way facilitates net carboxylation, and mechanisms for this could be visualized. Part, at least, of the low temperature effect should be so ascribed. Although less CO_2 is produced at low temperatures, its greater solubility then and equilibration with the atmosphere apparently lead to more CO_2 being held in the cell sap (Willaman and Brown 1930).

In postulating the involvement of an inhibitor which interacts with some promoting agent or system, Morley (1958) has taken into consideration findings by Loftus Hills (1942, 1944*b*) that high temperature during storage accelerates decline in dormancy, and that removal of the testa virtually abolishes dormancy. The seed storage data are not entirely internally consistent, and alternative explanations of the observations are possible. That removal of seed coats induces germination does not necessarily imply the removal of an inhibitor. This treatment widely brings about germination, and Toole *et al.* (1956) cite cases where inhibitors are not involved; but rather some other direct effect on the embryo. While the required characteristics of the promotive system could be met by the carboxylation step proposed here, the evidence for inhibitors is entirely inferential.

Irrespective of the number of pathways by which the germination of subterranean clover seeds may be accomplished, evidence exists for some distinct steps. The decline of a condition of "ultra-dormancy" (Section III(*b*)(ii)) indicates one. That the rates of CO₂-induced germination are similar, both where no ultra-dormancy existed, and where it had existed and disappeared, suggest this step to occur prior to any promotive process. The decline of this refractory condition cannot be accelerated by any of the dormancy-breaking treatments referred to here.

Also, the increase in rate of germination following pre-incubation (Section III(*b*)(iii)) indicates the accumulation of a reactant formed prior to C. Accumulation of such a precursor would favour carboxylation.

(*d*) Delayed Field Germination

The reasons should now be clear for the provisos attached to an earlier conclusion (Ballard 1958) that, because of the activity of soil CO₂, hard-seededness is of greater significance than dormancy in securing delayed germination in the field. The results of Section III(*b*)(ii) show that ultra-dormancy cannot be broken by either CO₂ or low temperature, so that passage out of dormancy would set the pace *if the seeds were soft*. Likewise, the effect of CO₂ is slight at 30°C—a temperature which may easily be attained in summer at soil surface.

However, on present evidence, it seems that only the most dormant varieties manifest this most refractory dormancy, and only in some seasons (or conditions of maturation). Also this type of dormancy appears to be of limited duration. For other strains, diurnal temperature fluctuations would be expected to allow some time for CO₂ action, and hence accumulation of C, albeit somewhat delayed.

Under these somewhat limited conditions, dormancy may control field germination at the peak of summer and for a brief period after seed maturation; but conservation of seed from season to season would be expected to be achieved via hard, and not dormant, seeds.

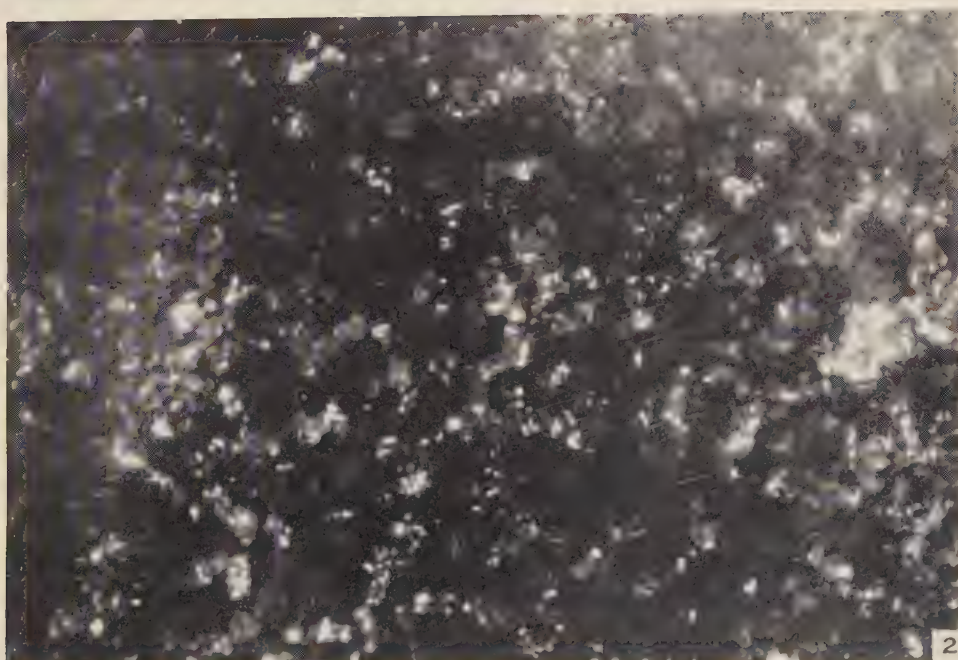
V. ACKNOWLEDGMENTS

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TRANSMISSION OF CAULIFLOWER MOSAIC VIRUS BY APHIDS



THE TRANSMISSION OF CAULIFLOWER MOSAIC VIRUS BY APHIDS

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Summary

Cauliflower mosaic virus (CIMV) has some characteristics of both non-persistent and persistent aphid-borne viruses. It has been shown that CIMV is a true non-persistent virus, and it is suggested that the unusual features of its transmission characteristics are due to its relative stability and its distribution in the tissues of the plant. These considerations lead to more precise definitions of the terms "persistent" and "non-persistent".

I. INTRODUCTION

Most of the phytopathogenic viruses transmitted by aphids may be classified as "non-persistent" or as "persistent" viruses. These terms, introduced by Watson and Roberts (1939), were subsequently somewhat modified in meaning (Watson 1946), and are now widely used. It is generally agreed that non-persistent viruses are transmitted by contaminated stylets, whereas persistent viruses are ingested, pass through the haemocoel, and are inoculated into a plant with the aphid saliva.

The range of characteristics of these two groups of viruses is considerable, so that it has been thought that certain viruses do not readily fit into either category. Sylvester (1956, 1958) has suggested that an intermediate category be established to include these, which he called "semi-persistent" viruses. He considered beet yellows virus to be a typical member of the group, and thought that dandelion yellow mosaic virus and cauliflower mosaic virus should probably also be included in it.

Cauliflower mosaic virus (CIMV) was first recognized by Tompkins (1937), or possibly earlier by European workers (see Klinkowski 1953). Early work on aphid transmission of CIMV seemed to indicate that it was a typical non-persistent virus. However, subsequent investigations (summarized by Broadbent 1956) have tended to show that it is atypical. Thus Hamlyn (1955) commented that "the ability to remain infective for at least 3 hours in the feeding vector distinguishes cauliflower mosaic virus sharply from non-persistent viruses studied previously". Chalfant and Chapman (1956) went further and stated that "transmission of Brassica virus 2 appears typically non-persistent by the green peach aphid, but shows some characteristics other than those of non-persistent types when transmitted by the cabbage aphid." Van Hoof (1954) concluded that "the results . . . would place cauliflower mosaic virus in the persistent viruses." Chalfant (1959), however, concluded that CIMV was non-persistent in both green peach aphid and cabbage aphid but that the virus was not carried on the stylets of the latter species after long feeds.

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In view of the interest of CIMV in any discussion of mechanisms of transmission of viruses by aphids we have investigated its transmission, and the results are reported in this paper. It is concluded that CIMV is a true non-persistent virus, and a revised description of this category is suggested.

II. MATERIALS AND METHODS

The virus was that used by Day and Venables (1960). It was maintained by mechanical or insect inoculation in turnip (*Brassica rapa* L. cv. Flat Express) which served both as a source plant and as a virus indicator. No local lesion host is available. In the mechanical inoculations the leaves were lightly dusted with carborundum. Small squares of organdie wetted with the liquids to be inoculated were gently rubbed over the leaves which were then washed with water. A few experiments included wild mustard (*Sisymbrium orientale* L.) as an indicator, and this species was shown to be roughly equivalent to turnip in sensitivity.

The aphids used were mature apterae of *Myzus persicae* (Sulz.) and *Hyadaphis* (*Brevicoryne*) *brassicae* (L.). The former were maintained on a variety of hosts including chinese cabbage, turnip, *Datura stramonium* L., and *Physalis floridana* Rydb. Colonies of *H. brassicae* were maintained on turnip. Both species were transferred from source to indicator plants by means of a fine camel-hair brush.

Indicator seedlings were grown in boxes 14 by 14 by 4 in., and generally used in the cotyledon or one-leaf stage. Symptoms of viral infection first appeared 14 days after inoculation, and for about 7–14 days thereafter.

Source plants invariably showed severe symptoms similar to those described by Broadbent (1956). The change in concentration of infective virus in infected plants with time was studied by bioassay by sampling at weekly intervals after mechanical inoculation. Infected leaves were harvested, treated as described in Table 1, and inoculated on to turnip seedlings. The results (Table 1) show that there is no marked change in virus content over a period of several weeks after symptoms have become marked.

Indicator plants after infection were kept in glass-houses which were regularly sprayed with insecticides.

III. EXPERIMENTAL RESULTS

(a) *Inoculation of CIMV into the Aphid Haemocoelae*

CIMV, purified by the method described by Day and Venables (1960), was inoculated by the technique used by Day (1955) into 32 mature apterous *M. persicae*. No infections resulted when these aphids were allowed to feed on indicator seedlings for a period of 3 days. Twenty-four hours after inoculation 24 aphids remained alive and 8 were living at the end of the 3-day test period. A similar experiment was performed using *H. brassicae*. The results were the same, but *H. brassicae* tolerated the inoculation less well than *M. persicae*, only 16 remaining alive after 24 hr and 6 at the end of the 3-day feeding period. The poor survival may have been due to the toxicity of the purified CIMV when injected, because survival was much better in aphids inoculated by the same methods and at about the same time with plant extracts infected with potato leaf roll virus.

Previous workers (Day 1955; Heinze 1955) have shown that blood from aphids infected with certain persistent viruses, when inoculated into non-infective aphids, caused them to become infective. Blood from 16 *H. brassicae* bred on CIMV-infected turnips was inoculated into 16 non-infective aphids. Each was then placed on separate indicator seedlings for 3 days. No infections resulted.

TABLE 1
EFFECT OF TIME AFTER INOCULATION ON YIELD OF CIMV FROM INFECTED LEAVES

Tissue harvested regardless of symptoms, washed, frozen overnight, thawed, macerated in 2 vol. 0.5M pH 7.8 potassium phosphate buffer, filtered, clarified (20 min at 12,000 *g*), filtered, and sedimented (90 min at 30,000 r.p.m. (No. 30 head, Spinco model L)). Pellet resuspended in 0.1M pH 7.8 buffer at rate of 0.5 ml buffer/g tissue extracted. Dilutions were inoculated 24 hr after harvest. Numbers of infections recorded out of 16 indicators inoculated

Time after Infection (days)	Infections at Dilution of:		
	1	1/10	1/100
21	7	3	1
28	16	6	1
35	14	8	4
42	15	15	2
50	13	14	2
57	14	9	3

(b) *Time Required for Transmission of CIMV*

One of the characteristics of the non-persistent viruses is that the transmission time (the period from the beginning of the acquisition feeding period to the completion of the transmission feeding period) requires only a few minutes. Day and Irzykiewicz (1954, Table 1) showed, in fact, that *M. persicae* and *H. brassicae* could transmit CIMV when the transmission time was as short as 83 sec. In a more extensive test *M. persicae* transmitted CIMV when the acquisition feeding period and the transmission feeding period was 30 and 40 sec, respectively. However, only one transmission occurred out of 19 tests. It is therefore concluded that *M. persicae* is capable of functioning as a vector when the transmission time is short, but that it is relatively inefficient under these conditions.

(c) *Acquisition and Persistence of Virus in Aphids*

(i) *Influence of Acquisition Feeding Time on Transmission of CIMV by M. persicae and H. brassicae.*—Two experiments were carried out to examine this aspect. In the first, individuals of both *M. persicae* and *H. brassicae*, previously allowed an acquisition feed in excess of 24 hr on infected turnips, were transferred to a series of five indicator plants allowing 5 min feeding time on each indicator. This was replicated 10 times for each species.

The second experiment was of the same design except that a feeding time of 5 min was allowed before transfer to a series of indicator plants.

The results of these experiments (Table 2) show: (1) no difference between short and long acquisition feeding periods with *M. persicae*, but suggest differences with *H. brassicae*; (2) no difference between the two species of vectors after short acquisition feeding periods; (3) the ability of both species to transmit without the intervention of an extrinsic incubation period; (4) the ability of both species to infect several plants in the series of transfers.

TABLE 2
CIMV INFECTIONS IN TURNIP SEEDLINGS BY SINGLE APHIDS MOVED TO FIVE SUCCESSIVE PLANTS
AFTER 5 MIN TRANSMISSION FEEDING PERIODS

Aphid Species	Acquisition Feeding Period	Infections per Plant					Total No. of Infections
		No. 1	No. 2	No. 3	No. 4	No. 5	
<i>M. persicae</i>	> 24 hr	1	1	0	0	1	3
<i>H. brassicae</i>	> 24 hr	2	5	2	4	2	15
<i>M. persicae</i>	5 min	2	0	1	2	0	5
<i>H. brassicae</i>	5 min	3	2	0	0	0	5

(ii) *Persistence of Virus in H. brassicae*.—Forty-two individuals of *H. brassicae* taken from a colony raised on infected turnips were each transferred to a series of five indicator seedlings allowing 24 hr feeding of each aphid on each indicator, thus covering a 5-day feeding period for each aphid. At each transfer, note was taken of cast skins to show when each aphid moulted. From the results of this experiment (Table 3) it will be observed that *H. brassicae* can retain CIMV for 3 days. This period of retention is longer than for any previously described non-persistent virus and is of about the same length of time for which a vector of beet yellows remains infective following a short acquisition feeding period. The data also show no instance of transmission of CIMV following a moult by the aphid vector. The conclusion that transmission of the virus did not occur following a moult was more conclusively demonstrated by the test reported by Day and Irzykiewicz (1954).

(d) *Distribution of CIMV in Plant Tissues*

The distribution of CIMV in tissues of infected turnip leaves was studied by the fluorescent antibody technique. Antiserum to virus purified by the method of Day and Venables (1960) was produced in rabbits. The inoculum consisted of 0.5 ml of the virus preparation diluted 1 in 4 in standard saline to which 2 ml of Freund's adjuvant was added. The mixture was homogenized for 5 min at maximum speed in a Virtis homogenizer. Two doses, each of 0.8 ml, were inoculated intramuscularly into the flanks of 3-month-old rabbits. Forty days later a booster injection consisting of 0.5 ml of virus solution and 1.5 ml standard saline was inoculated by the

same route. The rabbits were bled 5 days later and the globulin fraction was coupled with fluorescein isothiocyanate (Sylvana Chemical Co.). Pieces of leaves were embedded in 10% gelatine, and frozen in dry ice. Sections approximately 5μ thick were cut on a Cambridge rocking microtome by the method of Louis (1957). The sections were dried for 30 min on the slide and fixed in acetone for 5 min to remove most of the chlorophyll. A Zeiss fluorescence microscope was used with an Osram

TABLE 3
DISTRIBUTION OF INFECTIONS OF CIMV WHEN SINGLE INDIVIDUALS OF
H. BRASSICAE WERE MOVED DAILY TO HEALTHY TURNIP SEEDLINGS OVER A
5-DAY PERIOD

× = infections; M = moulted skin found. An additional 24 aphids did not transmit; four of these moulted during the 5-day period

Aphid	Day 1	Day 2	Day 3	Day 4	Day 5
1	×	×	×		
2	×				M
3			×	M	
4	×		×	M	
5	×	M			
6	×				
7	×		M		
8	×	×			
9	×	M			
10	×	×			
11	×	×	M		
12	×			M	
13	×	×M			
14	×	×	M		
15	×				
16	×	×			
17	×	×			
18	×				
Total	17/42	8/42	3/42	0/42	0/42
Number of aphids moulting	0	5	5	3	2

HBO 200 mercury vapour lamp. Photographs were taken on HP₃ (Ilford) 35-mm film. Antigen was readily localized in the sections and appropriate controls showed that staining was specific for CIMV. Uninfected leaves showed only the fluorescence of residual chlorophyll and of cell walls. Fluorescein-coupled vaccinia virus antibody produced in rabbits failed to stain sections of infected or control leaves. In an attempt to remove chlorophyll, leaves were soaked in acetone for 6 weeks. This treatment, however, removed all trace of specific staining as well as the characteristic fluorescence of chlorophyll.

Cotyledons mechanically inoculated 6 days previously with clarified sap from infected leaves showed very sparse specific staining. In systemically infected leaves, many cells from most leaf tissues contained antigen (Plate 1, Fig. 1). Only the

xylem vessels regularly failed to stain. A few phloem cells were intensely stained and in these the entire cytoplasm was strongly positive. Within individual epidermal mesophyll cells the fluorescence was confined to the cytoplasm. Neither nuclei nor chloroplasts were ever stained. The distribution of the virus in discrete aggregates may explain the irregularity in infection in aphids fed for short periods, because an aphid may probe one of these aggregates and become infectious, or the probe may

TABLE 4

HEAT STABILITY OF CIMV

180 g CIMV-infected plants were macerated with 360 ml 0.1M sodium phosphate (Sorensen's) buffer, pH 7.6, then filtered through organdie. 10-ml aliquots were heated for 5 min at the temperatures indicated. After clarification in the Servall centrifuge, each supernatant was inoculated on to 16 indicator seedlings

Temperature (°C)	Unheated	50	55	60	65	70	75	80
No. of infections	2	5	5	10	8	7	9	8

miss a virus aggregate completely. The greater efficiency of transmission by *H. brassicae* after longer feeds is explicable if this species is more efficient than *M. persicae* in locating the phloem, or if the stylets of *H. brassicae* penetrate cells more often than those of *M. persicae*, rather than penetrating through epidermal cell walls, which contained no stained antigen.

TABLE 5

LONGEVITY OF CIMV IN 0.1M SORESENSEN'S BUFFER, pH 7.0, AT 50°C
Number of infections per 16 indicators inoculated

Time (min)	No. of Infections	Time (min)	No. of Infections
0	8	15	14
5	8	20	13
10	13	30	12

The distribution of antigen within epidermal cells was studied in strips from the epidermis of infected turnip leaves. Strips from freshly cut leaves were soaked in solutions of the stain for 20 min, washed in several changes of water, and mounted in glycerol. No viral antigen was found in guard cells, but stained granules occurred in the majority of epidermal cells (Plate 1, Fig. 2). It seems likely that the inclusion bodies described by Rubio-Huertos (1950) are the granules which contain the viral antigen. Under the oil-immersion lens these granules were seen to have smooth contours and frequently consisted of several lobes. Three or more granules per cell

were frequently seen, but in many epidermal cells they were absent. In mesophyll cells adhering to the epidermal strips viral antigen occurred between but not in chloroplasts.

The distribution of antigen in the turnip leaf described above was compared with that of another CIMV strain (originally isolated from wild mustard). In this, the amount of virus in the epidermal layers appeared to be similar to that of the common strain. However, stainable antigen was virtually absent from the mesophyll and there was less in the conducting tissue than was observed in leaves infected by the common strain. This second type of distribution may be usual in non-persistent viruses.

(e) *Resistance of CIMV to Thermal Inactivation*

Most strains of CIMV have been reported by earlier workers to be relatively thermostable. Tables 4 and 5 illustrate that infectivity in the strain used is retained after treatment at 50°C for 30 min, or 80°C for 5 min. These results confirm that the common strain of CIMV is a relatively thermostable virus. This is of significance in the transmission of the virus.

It may be anticipated that transmission of the relatively thermolabile strain described by Wei *et al.* (1958) would differ from that of the strain used in the present work.

IV. DISCUSSION

Eleven characteristics of non-persistent and persistent viruses are tabulated in Table 6. In the final column is shown the category into which CIMV falls in respect to each criterion.

It will be observed that CIMV is a typical non-persistent virus in most respects, but that there are five characteristics in which it has some of the properties of a persistent virus. Each of these is explicable if CIMV is: (1) more stable than most other non-persistent viruses; (2) distributed in the plant tissues in a manner which differs from the distribution of most non-persistent viruses. It has been shown that CIMV is a stable virus both to thermal and chemical inactivation. Concerning the second point, Mulligan (1957) has evidence for the view that CIMV and cabbage black ring spot virus are differently distributed in leaves. This conclusion is borne out by the transmission data of Hamlyn (1955), and is suggested by the experiments using fluorescent-labelled antibody. This technique has been used on plant tissues previously only by Schramm and Röttger (1959) to study tobacco mosaic virus, and data on the distribution of aphid-borne viruses in leaf tissues is unfortunately limited. On the basis of experiments involving virus inactivation by ultraviolet light Bawden, Hamlyn, and Watson (1954) concluded that cabbage black ring spot virus and henbane mosaic virus were distributed mainly in the epidermal cells. However, Hitchborn (1958) concluded that potato virus Y, potato virus C, tobacco mosaic virus, and henbane mosaic virus were present in approximately equal amounts in mesophyll and in epidermal cells, and concluded that the ultraviolet inactivation experiments were of doubtful validity. These conclusions leave some of the aphid-transmission data unexplained, and further experiments using the fluorescent antibody method on a variety of plant viruses would be very desirable. In the

TABLE 6

CHARACTERISTICS OF APHID-BORNE VIRUSES AND BIOLOGICAL PROPERTIES OF CLMV

N = CLMV has the property of a non-persistent virus; P = CLMV has the property of a persistent virus; \pm = CLMV has some of the properties of both groups

Character- istic No.	Non-persistent Viruses	Persistent Viruses	Properties of CLMV*	Reference
1	Short transmission cycle (seconds)	Longer transmission cycle (hours)	N	Severin & Tompkins (1948); Van Hoof (1954)
2	No extrinsic incubation period	Often an extrinsic incubation period	N	All authors
3	Pre-acquisition starvation increases vector efficiency	No such effect	P	Martini (1956)
4	Short acquisition feeding periods more efficient than longer ones	Long acquisition feeding periods more efficient than short ones	\pm	This paper
5	Aphids generally capable of infecting one or few plants	Aphids capable of infecting many plants; often remain infectious for life	\pm	Van Hoof (1954); Hamlyn (1955); this paper
6	Virus not recoverable from haemolymph of vectors	Virus usually recoverable from haemolymph of vectors	N	This paper
7	Virus not transmissible by vector following a moult	Virus transmissible by vector following a moult	N	Day and Irzykiewicz (1954)
8	Vector not infective after inoculation of purified virus into haemocoel	Vector capable of becoming infective when purified virus is inoculated into haemocoel	N	This paper
9	Vector specificity not not marked	Vector specificity often marked	N	Day and Bennetts (1954)
10	Virus generally transmitted by mechanical inoculation of expressed sap	Not generally trans- mitted by mechanical inoculation	N	All authors
11	Virus generally affects mainly epidermal tissues	Virus generally affects conducting tissues	\pm	Mulligan (1957); but see also Hitchborn (1958)

absence of further data, the suggestion that CIMV is distributed in leaf tissues differently from that of some other non-persistent aphid-borne viruses remains a likely hypothesis. It should be stressed that no antigen could be seen in cell walls. If the suggestion of Van Hoof (1958) is correct, namely that aphids acquire virus mainly from the cell walls of epidermal cells, then the fluorescent antibody technique may not provide useful information about the distribution of aphid-accessible virus. Furthermore, Van Hoof's suggestion of the source of the infective virus may render Hitchborn's (1958) conclusions concerning the relative amounts of virus in different tissues irrelevant to the problem of aphid transmission.

Cauliflower mosaic virus can certainly be transmitted for longer periods following the acquisition feed than most non-persistent viruses. But this "persistence" is not a good criterion for placing a virus in either the persistent or the non-persistent category. For example, myxomatosis virus is apparently transmitted by a comparable mechanism to that by which the non-persistent aphid-borne viruses are transmitted, yet it persists for months on the mosquito mouthparts. In view of the thermal stability of CIMV it is not unexpected that aphid vectors can remain infective for periods longer than the vectors of more labile viruses.

Cauliflower mosaic virus is thus a true non-persistent virus. The two characteristics in which it is unusual are primarily the result of two properties, its stability and its distribution in the plant. Some of the differences observed between *M. persicae* and *H. brassicae* in their ability to transmit CIMV may result from differences in their feeding behaviour. Such differences are illustrated by the data of Day and Irzykiewicz (1953).

The above considerations assist in the selection of the best criteria for assigning any new aphid-borne virus to either the persistent or non-persistent category. Of the 11 characteristics listed in Table 6, it will be apparent that Nos. 6, 7, and 8 are those which distinguish unequivocally between the two types of transmission, whereas Nos. 3, 4, 5, and 11 are features dependent upon the stability and distribution of the virus rather than its mechanism of transmission.

The view has been held that the best criteria for placing a virus in the non-persistent category are the effect of pre-acquisition starvation on transmission efficiency and the greater efficiency in transmission following short feeding periods compared with long feeding periods. These criteria undoubtedly hold for many non-persistent viruses, but they do not hold for CIMV. If the aphid can acquire a virus from deeper tissues such as the phloem, then feeding periods in excess of 10 min will be necessary to reach the tissue (Roberts 1940), and this time will negate the effect of a pre-acquisition starvation period.

We are now able to define more precisely the terms "persistent" and "non-persistent" viruses. A persistent virus is one in which: (a) the transmission time is long; (b) the virus is recoverable from the haemolymph of a vector; (c) the virus is transmitted following the moult of a vector; and (d) the vector is capable of becoming infective when purified virus is inoculated into the haemocoel. A non-persistent virus is one in which (a) the transmission time is short; (b) the virus is not recoverable from the haemolymph; (c) the vector is not capable of transmitting following a moult; and (d) the vector does not become infective when purified virus is inoculated into the haemocoel.

Persistent viruses may behave in one of two ways. They may either multiply in the vector or they may pass through the vector unchanged. Those in the latter category often have some of the characteristics of non-persistent viruses. It would seem desirable to re-investigate other apparently anomalously transmitted viruses in the light of the above conclusions. It seems likely that the category of "semi-persistent" viruses will be found to be superfluous.

It should be mentioned that the determination of whether a virus is persistent or non-persistent may be of more than theoretical importance. Several investigators (see Broadbent 1957) have shown that it is sometimes possible to control persistent viruses by the application of systemic insecticides, but success has rarely attended attempts to reduce the occurrence of non-persistent viruses by these methods.

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EXPLANATION OF PLATE I

Parts of turnip leaves infected with CIMV, stained by the fluorescent antibody technique

- Fig. 1.—Transverse section of leaf. The light spots indicate the presence of viral antigen. Note that this is abundant in the epidermis and in parts of the vascular bundles. Less antigen is seen in the mesophyll. Sections from uninfected leaves show little except the red autofluorescence of chlorophyll which is barely recorded photographically. 10×ocular, 10×objective.
- Fig. 2.—Part of an epidermal strip. Specific fluorescence indicating the presence of viral antigen in granules, generally 1-3 per cell. Antigen cannot be detected in nuclei, cell walls, or in guard cells. The chloroplasts of these show the characteristic fluorescence of chlorophyll. Epidermal strips from uninfected plants show a few very small granules with the characteristic fluorescence of fluorescein.

ENVIRONMENT AND SPORULATION IN PHYTOPATHOGENIC FUNGI

II. CONIDIA FORMATION IN *PERONOSPORA TABACINA* ADAM AS A FUNCTION OF TEMPERATURE

By I. A. M. CRUICKSHANK*

[Manuscript received October 18, 1960]

Summary

Using a leaf-disk technique an analysis was carried out to study the effect of environmental temperature on the length of the incubation period and sporulation intensity of *P. tabacina*. An analysis was also made of the sporulation response to temperature treatments prior to and during sporulation. The following points were demonstrated:

- (1) Infected tobacco leaf is potentially capable of some sporulation from the 4th to the 17th days after infection. Maximum sporulation occurs 7–8 days after infection.
- (2) Sporulation of *P. tabacina* is characterized by an optimal temperature range of 15–23°C, a minimum temperature of 1–2°C, and a maximum temperature of 30°C.
- (3) The shape of the response curve is a characteristic of the fungus and independent of the condition of growth of the host plant.
- (4) The intensity of sporulation is dependent on the physiological state of the host plant, the stage of the incubation phase at which it occurs, and the isolate of *P. tabacina*.
- (5) The interaction between presporulation temperature and time affects sporulation intensity. Significant reduction in response occurs at high or low temperatures when the exposure times exceed 6–8 hr.

These results are discussed in relation to the epidemiology of blue mould.

I. INTRODUCTION

Clayton and Gaines (1933, 1945), Armstrong and Sumner (1935), and Dixon, McLean, and Wolf (1936), using plants grown under seed-bed, glass-house, and field conditions respectively, made observations on the effect of temperature on sporulation of *Peronospora tabacina* Adam. However, no systematic studies were carried out and no attempt was made to put the observations on a quantitative basis. Their values for the minimum, optimum, and maximum temperatures for sporulation appear to vary according to the locality, the isolate of the fungus, or the conditions under which the observations were made. Clayton and Gaines (1945) also observed and in part measured the effect of high day temperature on sporulation intensity but no detailed analysis of the response was attempted.

Since the overseas reports on the optimal temperature for sporulation of this fungus varied, and no Australian records were available, it was considered of interest to examine this and associated effects of temperature on sporulation, which might be

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of value in explaining some aspects of this phenomenon under field conditions. In this paper is presented a quantitative analysis of sporulation *in vivo* as a function of temperature. Firstly, the effect of the temperature factor in three environmental regimes on the length of the incubation phase of infection, and the intensity of sporulation with time, is studied; secondly, the effect of constant leaf-temperature levels during both presporulation and sporulation is reported; finally an analysis is presented of the effect on sporulation intensity of varying day and night leaf temperatures.

II. MATERIALS AND METHODS

An isolate of *P. tabacina* originally from the Ovens River Valley, Vic., and subsequently described as the Canberra isolate, was used in all experiments unless otherwise stated.

The host plant material (*Nicotiana tabacum* L. cv. Virginia Gold) and its preparation prior to sporulation studies were each similar to that described previously (Cruickshank 1958). The infected leaf-disk technique of Cruickshank and Müller (1957) was used to measure the sporulation intensity per unit area of infected leaf. A water-water system replaced the manitol-glycerol-water system to ensure that the diffusion pressure deficit and the relative humidity levels were constant and within the optimal range for maximum sporulation, irrespective of the temperature level. Six or eight leaf disk replications were used in all experiments to measure sporulation intensity and the results were statistically analysed after square-root transformation of the data. Within experiments, for ease of comparison, counts were converted to a 0-100 scale and the values termed the sporulation intensity index.

III. EXPERIMENTAL

(a) *Effect of Environmental Regime on Length of the Incubation Phase of Infection and the Intensity of Sporulation with Time*

Groups of six tobacco plants were conditioned for 2 weeks to each of the following environmental regimes:

Regime	Day Temp. (°C)	Night Temp. (°C)	Relative Humidity (%)	
	(0830-1630 hr)	(1630-0830 hr)	(day)	(night)
I	20	15	c. 48	50-70
II	25	18	c. 35	35-150
III	28	24	c. 40	42-50

The plants were removed for inoculation and after 24 hr returned to their respective environmental regimes. Leaf disk samples for sporulation tests were taken daily from each plant at 1700 hr and incubated overnight at 20°C as described above.

The results of this time course experiment, presented in Figure 1, show that the ability of *P. tabacina* to sporulate rose from zero on the 3rd-5th day to a maximum on the 7th-8th day after inoculation. Its subsequent capacity to sporulate fell off with time. Under each of the three environments used the observations showed that while

green or chlorotic leaf areas remained, *P. tabacina* retained some capacity to sporulate. In this series of determinations the level did not fall below approximately 25% of the maximum level.

The individual sporulation capacities were determined by the environmental regimes under which the host plants were growing. The first signs of sporulation occurred under regime III on the 4th day after inoculation, and on the 6th day under regimes I and II. There was, however, a significant difference ($P < 0.001$) in sporulation level between the latter two regimes at this stage. Sporulation reached its maximum level in regime III on the 7th day. At this time there was a significant difference ($P < 0.001$) between the sporulation responses under regimes I and II and under regime III. Eight days were required for maximum sporulation under regimes I and II and, at this stage, when maximum sporulation was occurring under all three environments, there was a significant difference ($P < 0.001$) in sporulation intensity between regimes I and III but not between regimes II and III. This latter relationship was maintained throughout the decline in sporulation capacity over the balance of the experiment.

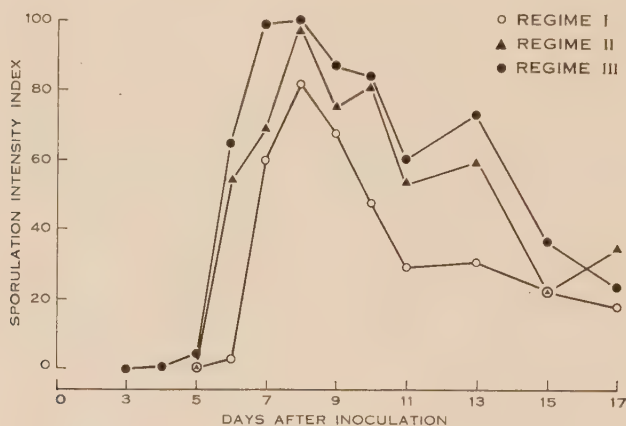


Fig. 1.—Time course of sporulation intensity of *P. tabacina* in leaf disks.

(b) Effect of Leaf Temperature on Sporulation Response

In this group of experiments the change in sporulation intensity with temperature was studied on two sets of material. In the first experiment (b,i) the fungal isolate was held constant. The responses of *P. tabacina*, infecting tobacco leaves grown in each of the three controlled environmental regimes, were compared. In the second experiment (b,ii) the environment was held constant (regime II) and the sporulation response of multispore isolates of *P. tabacina* from Parada, N.Qld., Canberra (originally Ovens River Valley, Vic.), and Manjimup, W.A., were compared. All experiments were repeated, and sporulation patterns confirmed.

In each experiment the leaf disk samples were cut from plant replicates in each treatment 8 days after inoculation. They were incubated in a series of unlighted

incubators set at temperatures ranging from 1 to 30°C. After 24 hr the disks were harvested and the sporulation intensity measured. Results of experiments (b,i) and (b,ii) are presented in Tables 1 and 2 respectively.

TABLE 1
COMPARISON OF SPORULATION OF *P. TABACINA* ON LEAF DISKS FROM TOBACCO
PLANTS GROWN IN THREE ENVIRONMENTS

Incubation Temp. (°C)	Mean Sporulation Intensity Index		
	Regime I*	Regime II	Regime III
1.5	0.05	0.01	0.00
4.0	0.39	0.39	0.32
8.1	6.72	6.89	11.6
15.0	26.62	36.82	71.19
17.0	32.04	33.62	81.74
19.6	29.69	56.94	100.00
22.9	28.05	40.02	88.25
26.4	0.38	0.57	1.18
29.7	0.00	0.00	0.06

* Environmental regimes given on p. 199.

An analysis of the sporulation data from both experiments gave a unimodal response curve strongly skewed to the right if increasing temperature is plotted as the

TABLE 2
COMPARISON OF SPORULATION OF THREE ISOLATES OF *P. TABACINA* ON LEAF DISKS
FROM TOBACCO PLANTS GROWN IN THE SAME ENVIRONMENT (REGIME II)

Incubation Temp. (°C)	Mean Sporulation Intensity Index		
	Parada Isolate	Canberra Isolate	Manjimup Isolate
1.7	0.09	0.15	0.44
4.0	1.03	6.76	1.84
8.5	21.14	35.16	16.00
15.0	38.95	85.12	48.22
18.5	39.04	95.87	52.55
20.2	44.23	100.00	54.96
23.6	36.90	80.61	45.82
26.9	0.00	1.61	0.02
30.2	0.00	0.00	0.00

horizontal ordinate (cf. Cochrane 1958). The minimum, optimal, and maximum temperatures were, 1-2, 15-23, and 30°C respectively.

Sporulation intensities of *P. tabacina* growing in leaves of plants grown under three environmental regimes are given in Table 1. Over the temperature range 15–22.9°C the intensities increase from regimes I through II to III, all differences at the same temperature being significant ($P < 0.05$ at least) except between regimes I and II at 17°C. Reference to Figure 1, which shows the relative daily sporulation patterns of the Canberra isolate of *P. tabacina* at 20°C, confirms the above relationship between the sporulation intensity indices of *P. tabacina* under regimes I and III over the major portion of the period the sporulation responses were tested. The situation in relation to *P. tabacina* in regime II is less clear. An intermediate sporulation intensity is maintained but the level of significant difference from the other two regimes is variable.

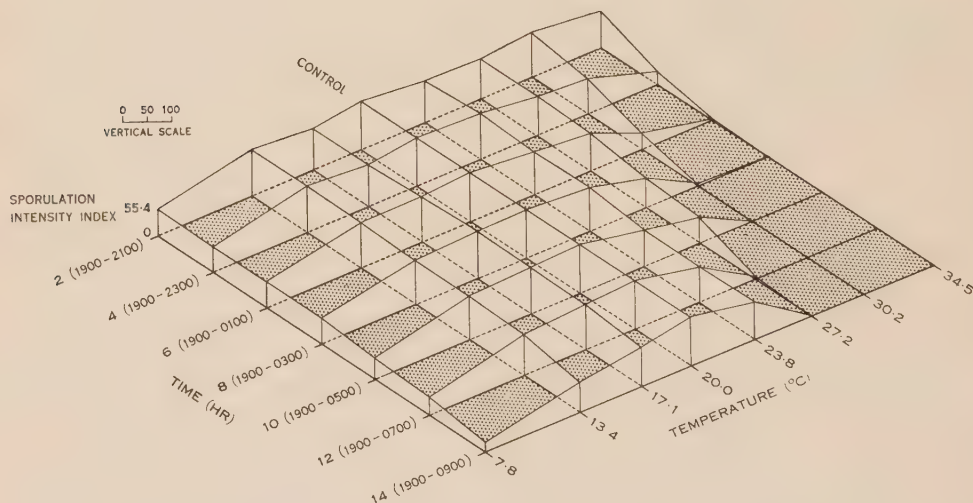


Fig. 2.—Relationship between night temperature, its duration, and sporulation intensity (night temperature constant, c. 20°C).

The comparison of the three isolates of *P. tabacina* showed that the Canberra isolate sporulated with approximately twice the intensity of the isolates from either Manjimup or Parada. A subsequent comparison of the Canberra isolate with a recent isolate from the Ovens River Valley has shown no difference in sporulation behaviour. It is concluded from these experiments that although the pattern of change in sporulation response with temperature was independent of the conditions of growth or the geographical source of the pathogen, the actual levels of sporulation intensity at any particular temperature appear to be closely related to these factors.

(c) *Effect of Various Temperature and Time Combinations on Sporulation Response*

(i) *Day Temperature Constant and Night Temperature Varied.*—Leaf disks were cut from infected host tissues which had been growing under regime I (day temperature 20°C) and incubated at each of the following temperatures: 7.8, 13.4, 17.1, 23.8, 27.2, 30.2, and 34.5°C. In the first study (results shown in Fig. 2) the treatments

were initiated at 1900 hr. Disk samples from each temperature were removed after 2, 4, 6, 8, 10, 12, and 14 hr and transferred to an incubator at 20°C until 0900 hr.

In the second experiment (illustrated in Fig. 3) the same temperature levels were used but the reverse time procedure was followed, viz. all leaf disks were placed in the 20°C incubator at 1900 hr and progressively transferred at 2-hourly intervals to the above temperature conditions where they remained until 0900 hr the following morning. In each series, the disks were killed at 0900 hr by addition of a drop of 1% formalin and then transferred to 50% ethanol as described in the technique referred to above.

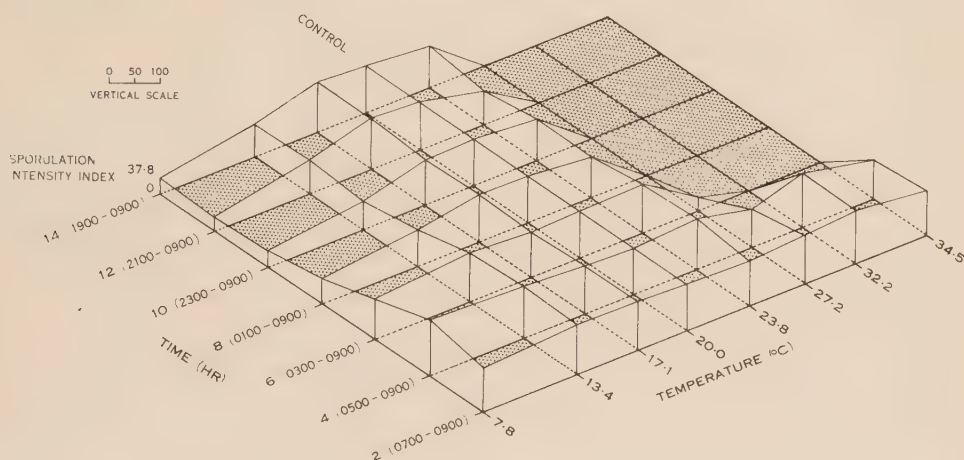


Fig. 3.—Relationship between night temperature, its duration, and sporulation intensity (night temperature constant, c. 20°C).

(ii) *Night Temperature Constant and Day Temperature Varied.*—In this experiment (results shown in Fig. 4) leaf disks were incubated at 8.5, 13.8, 17.6, 24.3, 27.8, 31.3, and 35.1°C for 2, 4, 6, 8, and 10 hr, starting from 0930 hr. After exposure the disks were transferred to a common incubator at 20°C for the balance of the 24-hr experimental period. The disks were then harvested and the sporulation intensities measured.

In each of the three experiments in this section one complete set of disks (times \times replicates) were incubated at 20°C over the full experimental period to serve as controls.

The three-dimensional diagrams (Figs. 2, 3, and 4) represent in each case essentially the effect of superimposing fairly small temperature treatments for either increasing or decreasing intervals of time on a basic 20°C grid. In the two experiments where the night temperature treatments were changing with time, the times included the normal sporulation period—in this case 0300–0500 hr. On the other hand, in the experiment in which night temperature was held constant, the effect of day temperature was reflected in the sporulation response independently of the specific sporulation period.

Taking the response at 20°C as control and comparing other responses with it the following points can be demonstrated. In the first experiment (Fig. 2) temperatures 7.8 and 34.5°C significantly ($P < 0.01$) depressed the sporulation response over the whole time range. The effect of high temperature was, however, much greater than that of low temperature; for example, the effect of the 13.4°C treatment only reached significance ($P < 0.01$) after 14 hr exposure, viz. from 1900 to 0900 hr, while the effect of the 27.2 and 30.2°C treatments, on the other hand, significantly depressed sporulation in all but the 2-hr exposures. In the second experiment (Fig. 3) the 2-hr exposure occurred between 0700 and 0900 hr. At this time of day neither the lowest two (7.8 and 13.4°C) temperature treatments nor the highest three

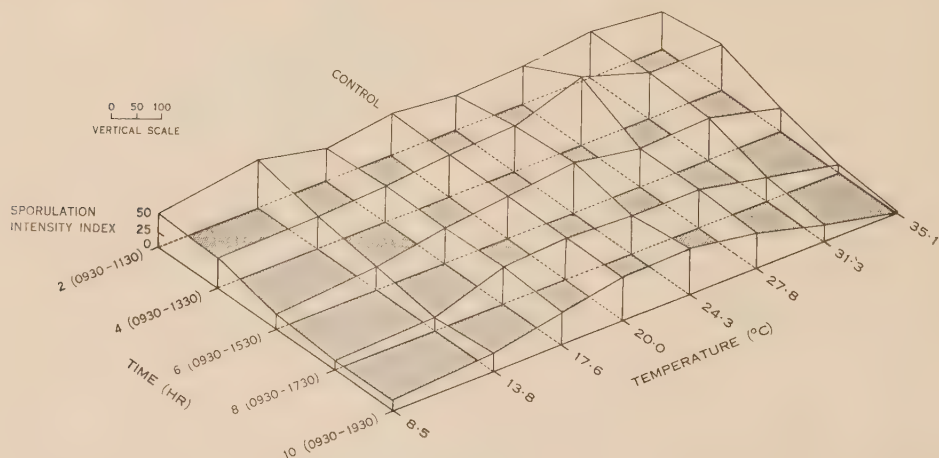


Fig. 4.—Relationship between day temperature, its duration, and sporulation intensity (night temperature constant, 20°C).

temperature treatments (27.2, 30.2, and 34.5°C) had any effect on sporulation. As the time of initiation of the lower treatments, however, receded from 0500 to 1900 hr the sporulation response was progressively reduced at these temperatures. The effect at the higher temperatures was more rapid. Sporulation dropped to zero where the highest temperature treatments (32.2–34.5°C) were initiated at 0300 hr. The same effect was produced at 27.2°C when the treatment was initiated at 0100 hr. Over the intermediate temperature range 17.1–23.8°C in both experiments treatment had no effect on the sporulation response.

In the third experiment (Fig. 4) no treatment of 2 hr significantly affected the sporulation rate; however, the 8.5 and 13.8°C treatments after 4 hr significantly ($P < 0.01$) reduced sporulation intensity and this effect increased with time of exposure to the treatment. The 17.6 and 24.3°C treatments did not significantly effect the sporulation response. At 27.8°C there was a significant ($P < 0.001$) increase in intensity after 4 hr, but with further increase in time of exposure the response fell and became not significantly different from the control. Treatments for 8 and 6 hr at 31.3 and 35.1°C, respectively, reduced the sporulation response significantly ($P < 0.001$) and this effect increased with time of exposure.

(d) *Comparison of Monthly Mean Temperatures in Tobacco-growing Districts*

Data on the mean maximum and minimum temperatures for several tobacco-growing districts in Australia were obtained. However, only in two of the recording stations were the records available over long periods (30 years). These data are plotted in Figure 5 alongside two similar sets of data relating to two tobacco-growing districts in the United States (McGrath and Miller 1958). These data alone do not, in terms of the experimentally determined temperature responses, suggest differences between tobacco districts in Australia and the United States sufficient to explain the differences in incidence and severity of blue mould which occur between these two countries.

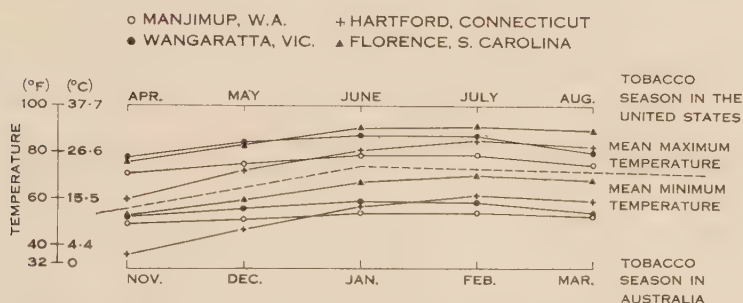


Fig. 5.—Comparison of long-term mean maximum and minimum temperatures between two tobacco-growing districts in the United States and Australia.

IV. DISCUSSION

Sporulation as a function of temperature has been studied extensively *in vitro* and has been recently reviewed by Hawker (1957). *In vivo* studies of the sporulation response of phytopathogenic fungi to individual factors in the environment have received much less attention. Most *in vivo* studies have been limited to field studies in which semiquantitative observational methods have been employed.

Previous studies of this problem in relation to *P. tabacina* have been concerned primarily with devising control measures rather than with the behaviour of *P. tabacina*. The leaf-disk technique used here has enabled detailed *in vivo* studies to be carried out quantitatively. It has also allowed not only the effect of the ambient or environmental temperatures on sporulation to be considered but also the measurement of the effect of actual leaf temperature.

Several authors have studied the sporulation of *P. tabacina* under constant temperature conditions. Reported optimum temperatures range from 13.3 (Dixon, McLean, and Wolf 1936) to 21°C (Armstrong and Sumner 1935). In the present studies in which glass-house grown leaf material was used as the host substrate there was no significant difference in sporulation intensity over the temperature range 15–23°C. The host material studied here represents plants grown under different temperature regimes. The isolates of *P. tabacina* studied represent isolates of the fungus from widely separated geographical regions. The similarity of the sporulation response with increase in temperature suggests that *in vivo*, as *in vitro* in the case of facultative fungi,

the slope of the response curve is a characteristic of the fungus species and is independent of the host substrate. The intensity level of sporulation at any given temperature is, however, a function of the physiology of the host and the strain of the fungus. Although the Canberra isolate of *P. tabacina* was readily distinguished from those from Parada and Manjimup on the basis of its greater sporulation capacity it could not be distinguished from a recent isolate from Ovens River Valley. It thus appears that several years' (1954-1960) isolation and experimental use have not been sufficient to modify this characteristic.

Temperature affects both the length of the presporulation phase of vegetative growth of *P. tabacina* in the host tissues and the intensity of the sporulation response. Both these factors may be of considerable significance in terms of the epidemiology of blue mould. On account of their low night humidities the conditions of the regimes studied were unfavourable for sporulation. They were, however, satisfactory for vegetative growth. Potentially, the fungus was able, under these conditions, to sporulate on the 4th-6th day after inoculation, while maximum sporulation was possible after 7-8 days. Sporulation could be inhibited by high temperatures or low night humidities (Cruickshank 1958). The inhibition was, however, only temporary as the fungus remained potentially capable of sporulation for at least 10 days under all three environmental regimes. Thus, although the maximum sporulation for a particular lesion may not occur due to favourable conditions not coinciding with the 7th-8th day after infection, the lesion nevertheless is capable of producing conidia in considerable quantities providing favourable conditions occur some time from the 6th to at least the 17th day after infection. Under conditions of less severe infection than those used in the experiments described in this paper, the period over which sporulation could occur may be considerably longer than 10 days.

Under field conditions temperatures rarely remain constant for long periods of time. Although it is of interest to know the cardinal temperatures for sporulation, it is much more important from the epidemiological point of view to know the expected response for given time and temperature combinations. The situations chosen here were very simplified in relation to the fluctuating natural environment. They do, however, emphasize that the duration of exposure to a given temperature, and not temperature alone, decides the final sporulation level. This data, particularly in relation to high day temperatures, could possibly be of use in blue mould forecasting.

In nature the following generalized environments are possible in terms of temperature: cold days and cold nights; warm days and cool nights; hot days and cool nights; hot days and hot nights. In terms of the time and temperature combinations reported above hot days followed by either hot nights or cool nights would be least favourable for sporulation. Cold days and cold nights would also depress spore production, while warm days and cool nights, on the other hand, would favour maximum levels of sporulation. The data shown in Figure 5 do not indicate large differences in the mean temperatures between tobacco districts in Australia and the United States. The mean minimum temperatures (and therefore night temperatures) in general fall almost within the optimal temperature range for sporulation. The mean maximum temperatures, on the other hand, would inhibit sporulation only if maintained over extended daily periods. In Australia rapid changes in temperature frequently occur even in the

summer months. In the United States uniformly high summer temperatures are normally experienced. The dependence of sporulation not only on temperature but also its duration could be of significance in explaining why blue mould is more serious as a field disease in Australia than in the United States.

Within Australia differentiation with respect to pathogenicity and reproductive capacity between isolates of *P. tabacina* has been reported by Wark *et al.* (1960). Similar differences to those demonstrated may be true of ecotypes of *P. tabacina* in different countries. These differences may also be important in influencing the epidemiology of blue mould.

V. ACKNOWLEDGMENT

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DISSEMINATION OF CONIDIA OF *PERONOSPORA TABACINA* ADAM

By A. V. HILL*

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Summary

Conidia of *P. tabacina* in the atmosphere of tobacco plots and glass-houses were trapped by means of a Hirst spore trap.

Greatest numbers of conidia were trapped at approximately the same time each day. In the field the number commenced to increase between 0545 and 0715 hr, reached a maximum between 1000 and 1100 hr, decreased to a few between 1430 and 1630 hr, and remained low until the next morning. In the glass-house, maximum and minimum numbers usually occurred later in the day.

The number of conidia trapped during periods of increasing leaf movement was less than during the regular daily dispersal period.

The daily cycle of dissemination is considered to be due to the collapse of the conidiophore as a consequence of the withdrawal of water into the mycelium in the leaf. In so far as atmospheric temperature, humidity, and light are factors effecting changes in leaf turgidity, they are also factors in spore dispersal.

The daily cycle of conidial discharge with rising temperature, increasing insolation, and decreasing humidity is considered to provide for distribution of conidia to nearby plants. A more widespread distribution of conidia would be obtained with leaf movement in a turbulent atmosphere at any time of the day, but especially if such conditions occur during the normal daily cycle.

Conidia trapped during each daily dispersal period were viable even though humidities at the time of trapping were as low as 35%.

I. INTRODUCTION

In the course of studies on the diurnal change in atmospheric spore content Hirst (1953, 1958) concluded that dispersal of conidia of *Phytophthora infestans* (Mont.) de Bary commenced about 0700 hr, reached a peak at about 1100 hr, and few conidia were found after 1900 hr. Experiments in a wind tunnel showed that conidia were not liberated from leaves by wet or dry gales but were removed by wetting, rapid changes in relative humidity, or strong light. Radiation, possibly associated with rapid humidity changes close to the surface of the leaf, was suggested as a major factor in dispersal of conidia from leaves (Hirst 1958). Dispersal of conidia of the closely related pathogen *Peronospora tabacina* Adam from detached leaf disks was brought about by mechanical shock or reduction of ambient humidity (Cruickshank 1958). In field studies, Waggoner and Taylor (1958) found that conidia of *P. tabacina* were present in the atmosphere in maximum numbers on clear days at approximately 0500–0600 hr or earlier, and on cloudy days at 0830–1030 hr. With both of the above pathogens, spore discharge was considered to be brought about by twisting movements of conidiophores exposed to rapidly changing, and decreasing, humidity (Pinckard 1942; Hirst 1953).

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According to the above data the thin-walled conidia of both pathogens are dispersed at times of the day when atmospheric conditions are apparently becoming progressively less favourable for their survival. Crosier (1934) showed that conidia of *Phytophthora infestans* survive for less than 8 hr at 90% humidity and for only a few hours at lower humidities. However, conidia of *Peronospora tabacina* can survive for much longer periods (Angell and Hill 1932). Increasing temperature and insolation do not favour survival of conidia (Angell and Hill 1932) and disappearance of the dew and water drops, necessary for germination, from leaves would prevent the establishment of new infections. Nevertheless both pathogens cause damaging epiphytotics.

This paper is concerned with the numbers of conidia of *P. tabacina* in the atmosphere in the vicinity of sporulating leaves of tobacco plants grown in the glass-house and field at Canberra.

II. METHODS

Conidia of *P. tabacina* were trapped over three years using the methods of Hirst (1952) in which a slide smeared with petroleum jelly moves past an orifice 14 by 2 mm at the rate of 2 mm/hr. Counts of conidia trapped on slides over a 24-hr period were made at intervals of 2 mm on traverses at right angles to the length of the slide. A record of one conidium per hour represented a mean density of approximately 200 conidia per m³ of air throughout a period of 1 hr. In practice the actual number passing through the m³ during the hour would usually be much greater, because, as shown later, maintenance of a mean density involved continuous replacement of conidia lost to the surrounding atmosphere. Usually the mean of the numbers of conidia recorded for adjoining hours was plotted with temperature and humidity recorded by thermohygrographs placed near the trap opening. Rainfall, light, and air movement, were recorded as required.

When conidia were trapped for short intervals of time, successive collections were made on a newly exposed section of the slide.

In the glass-house experiments, sporulation, usually on plants 40–60 cm in height, was obtained by placing them in a saturated atmosphere about 7 days after inoculation. After sporulation, humidity was usually maintained at a level too low for further production of conidia on subsequent days. In the glass-house, sporulation was not observed at ambient humidities below 90%, and in the field it was very limited or did not occur below humidities of approximately 95%.

The effect of light and darkness on numbers of conidia was examined in a room fitted with fluorescent lights, a low-speed electric fan operating continuously at floor level, and a thermostatically controlled electric heater of 200 W. Illumination at the level of the upper leaves was approximately 300 f.c. Leaf sporulation area was of the order of 1200 cm².

In preliminary tests in the field, spore traps were placed between the rows in two plots each of 270 tobacco plants grown in the open and under lath shading respectively, the trap openings being 46 cm above soil level. Similar results were obtained with both traps and subsequent trapping was done amongst plants grown under lath shade. In 1957, the plants were approximately 150 cm in height and in

the two following years about 48 cm in height when trapping commenced. Temperature and humidity records at the height of a few centimetres below the level of the trap opening were obtained from thermohygrographs in standard Stevenson meteorological screens.

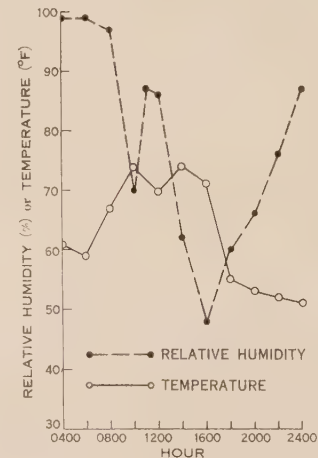
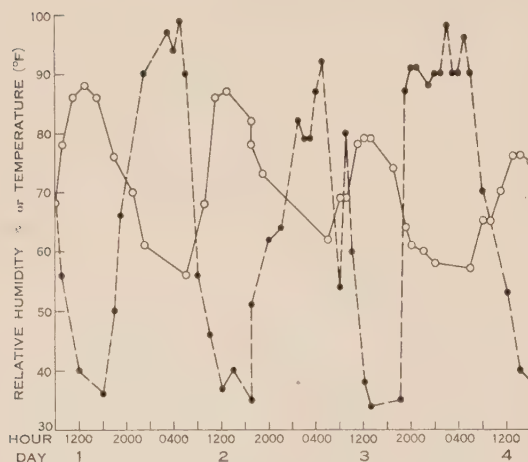
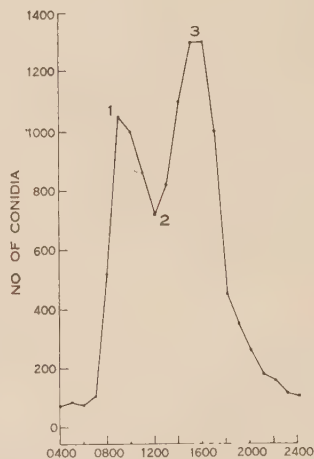
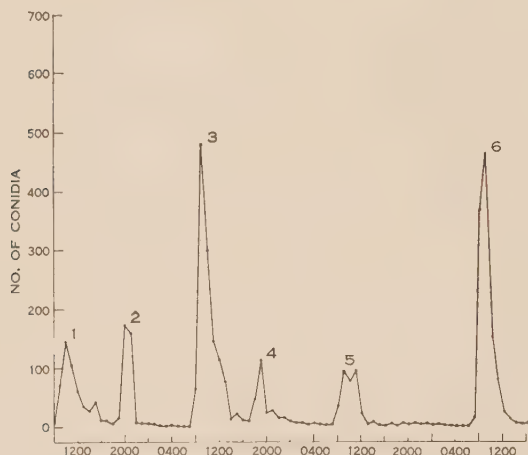


Fig. 1

Fig. 2

Fig. 1.—Dissemination of conidia of *P. tabacina* in the field to show at 1, 3, 5, 6, peaks of diurnal dissemination; at 2, the effect of 0.01 in. of rain; and at 4, the effect of wind.

Fig. 2.—Dissemination of conidia of *P. tabacina* in the field to show at 1, peak of diurnal dissemination; 2, effect of 0.05 in. of rain between 1100 and 1200 hr followed by 3, renewed dissemination with peak at 1500–1600 hr.

Information on viability of conidia trapped was obtained by placing the slides, after the conidia were counted, in moist petri dishes for 24 hr. The percentage germination recorded for any particular time was the maximum observed in the conidia trapped at that time.

III. RESULTS

(a) *Leaf Movement as a Factor in Dissemination of Conidia*

In the field, light showers of rain, often accompanied by gusts of wind, caused the number of conidia trapped per hour to change (e.g. from 12 to 166, and from 20 to 138) on one-third of the nights during the sporulation period, the actual number

TABLE 1
DISSEMINATION OF CONIDIA IN THE FIELD IN THREE SEASONS

Year	No. of Days of Record	Normal Dissemination		Abnormal Dissemination		
		Mean Times (hr)	Mean Time of Max. Numbers (hr)	No. of Days	Time of Day (hr)	No. of Hours per Day
1957	12	0715-1437	1100	4	1700-0400	2-4
1958	17	0546-1625	1042	7	1700-0600	1-2
1959	15	0628-1630	0956	4	1400-2400	1-2

trapped being less than 10% of the number caught during the previous or the following morning (Fig. 1, reference points 2 and 4). Similar results were obtained when

TABLE 2
EFFECT OF LEAF MOVEMENT ON NUMBER OF CONIDIA TRAPPED

Numbers trapped over a period of 5 min and with 5 min rest interval between collections. For all treatments, temperatures were in the range 64-70°F and humidities in the range 76-81%

Treatment	No. of Conidia Trapped			
	10-5 min before Treatment	During Treatment Period	5-10 min after Treatment	15-20 min after Treatment
Air movement by electric fan*	7	76	29	9
Plant movement by blows on stem†	2	123	20	2
Falling fine sand†	29	755	220	40
Falling water drops†	25	480	74	21

* Fan used during treatment period of 2 min.

† Fan used to give slight air movement during each collecting period.

leaves were disturbed accidentally. The occurrence of short periods of dissemination associated with wind storms during late afternoon and night in three seasons in the field is shown in Table 1.

The actions of various natural factors causing leaf movement were simulated, under glass-house conditions, by subjecting similar lots of sporulating plants to air movement, falling water drops, falling fine sand, and vigorous stem movements. The results (Table 2) show that conidia became airborne as a result of leaf movement, and that a sharp rise and fall in numbers could occur within a few minutes. In other experiments few conidia became airborne as a result of leaf movement in a nearly saturated atmosphere.

TABLE 3

EFFECT OF AIR MOVEMENT ON NUMBERS OF CONIDIA TRAPPED

Numbers trapped in a glass-house from two lots of plants (A and B) during periods of 5 min and with rest intervals of 5 min between collections. For all treatments, temperatures were in the range 65–70°F and humidities in the range 85–91%

Air Movement (ft/min)	No. of Conidia Trapped							
	10–5 min before Treatment		During Treatment		5–10 min after Treatment		15–20 min after Treatment	
	A	B	A	B	A	B	A	B
30	1	1	3	1	1	1	1	1
75	1	1	13	120	3	—	1	—
90	1	1	14	53	2	4	1	1
105	1	—	10	75	1	25	1	4
150	1	4	5	145	1	33	1	4
230	1	4	15	104	3	30	1	5
450	1	5	21	250	13	5	2	2

Additional information on the effect of leaf movement due to wind for periods of 2–5 min was obtained in glass-house experiments. Results from two lots of plants, A and B, subjected for periods of 5 min to several rates of air movement by means of an electric fan are shown in Table 3. The greatest number of conidia was trapped within the first few minutes after the air movement was increased and thereafter a low number during the period of constant wind speed. If the wind speed was increased the pattern was repeated. The rapid dispersal and loss of conidia from the vicinity of the trap was shown in the comparison of the numbers trapped during and after leaf movements (Tables 1–3). There was a return to the normal air content of the glass-house within a few minutes after treatment ceased, a similar picture to that observed in the field. From this it was evident that the conidia became airborne as a consequence of the treatment.

(b) Diurnal Periodicity

Continuous trapping in the field and in the glass-house showed that there was a well-defined daily periodicity in atmospheric content of conidia (Table 4; Fig. 1, reference points 1, 3, 5, and 6). For all experiments the mean time at which the

numbers of conidia trapped commenced to increase was between 0530 and 0839 hr. Greatest numbers occurred between 0956 and 1342 hr and numbers decreased to a few by 1437–1930 hr or occasionally later. The number of conidia increased over periods of 3–7 hr and the total time that relatively high numbers were present varied from 7·3 to 18·5 hr. When very large numbers of conidia were present on the leaves relatively large numbers were trapped during one and sometimes two nights.

The rise in numbers of conidia trapped commenced at or subsequent to sunrise (about 0715 hr in June, 0450 hr in December) when temperatures were expected to rise and humidities to decline. However, periodicity was maintained with little or no change in humidity and with only small changes in temperature (Table 4). The results show that it was not necessary for the overnight humidity to be high for dissemination to occur on the following day. The peak in the number of conidia trapped occurred long before the daily maximum temperature or minimum humidity was reached.

Diurnal periodicity did not occur in darkness or in continuous light but it could be induced by alternation of light and dark or by an increase in temperature except when humidity was maintained at near saturation. In the glass-house, diurnal periodicity from the same plants was maintained for up to 19 days, comparative numbers of conidia per day varying from 430 to 7500. Humidity subsequent to the fourth day after the occurrence of sporulation was below 80%, the average being in the range 60–70%. In this and other experiments similar results were obtained both with and without continuous and uniform air movement.

Under field conditions continuous trapping showed that conidia were present in the atmosphere every hour for up to 11 days, but very few were present at near-saturation humidities. The longest period of near-saturation humidity following sporulation was 28 hr, and there were many shorter periods, during which none to few conidia were trapped. Such periods were associated with low light intensity or darkness. The numbers of conidia trapped in three seasons during the hours of greatest dissemination are shown as totals of four one-hourly counts commencing at 0830 (Table 5). In 1957, numbers increased daily to a maximum on the fifth day following several nights of high humidity whereas in 1958, non-sporulating lesions increased in number over a period of 3 days and 3 nights of low humidity before conidia were produced and maximum numbers were trapped on the first day of sporulation.

Maximum numbers of conidia were recorded at approximately the same time each day despite wide differences in weather conditions. The times of maxima for Canberra were compared with those recorded in Connecticut, U.S.A., by Waggoner and Taylor (1958) and with similar data for *Phytophthora infestans* in England (Hirst 1953) and related to time of sunrise. The number of hours from sunrise required for maximum number of conidia to be recorded in each locality was similar. This time interval did not differ greatly from that recorded at Canberra for a wide range of glass-house conditions.

(c) *Mechanism of Diurnal Periodicity*

Results from experiments on leaf movement showed that conidia could be detached from conidiophores during violent leaf movement. However, large numbers

TABLE 4
DIURNAL CYCLE OF DISSEMINATION OF CONIDIA
Times, relative humidities, and temperatures are mean for the stated period. G — glass-house; CG — controlled temperature glass-house; F — field (three seasons)

Period of Test (days) and place	Time of Cycle (hr)			Relative Humidity (%) at Time of			Relative Humidity (%)		Temperature (°F) at Time of			Temperature (°F)	
	Start	Maximum	End	Start	Maximum	End	Highest	Lowest	Start	Maximum	End	Lowest	Highest
14 G	0824	1300	1930	84.3	76.6	82.4	92.9	69.3	58.3	66.3	69.8	55.9	72.7
8 G	0712	1342	0148	62.3	62.0	61.6	66.1	58.9	65.4	76.0	68.3	65.0	77.4
12 G	0636	1012	1514	88.3	83.8	83.4	89.6	80.0	60.3	67.9	70.4	57.8	73.2
17 G	0530	1230	1912	74.7	67.3	71.6	76.7	64.7	72.4	78.2	73.2	61.2	80.9
11 CG	0652	1052	1518	55.3	50.4	56.4	69.4	47.6	58.1	70.0	67.7	58.0	70.6
14 CG	0753	1108	1538	41.5	40.0	48.3	50.3	39.1	68.5	80.0	73.5	65.5	80.4
12 CG	0839	1211	1600	51.0	47.2	48.9	55.8	41.1	77.4	86.0	81.5	71.0	87.8
20 CG	0712	1012	1554	58.6	57.9	57.2	62.2	53.9	72.2	82.9	84.1	70.4	89.2
28 CG	0700	1333	2322	72.0	51.4	70.9	79.9	43.4	72.2	85.3	74.0	67.7	94.1
14 F	0715	1100	1437	88.9	55.0	48.0	97.8	41.6	56.5	68.4	75.0	55.9	76.3
21 F	0546	1042	1625	92.6	65.4	61.1	94.5	47.3	59.1	70.9	78.2	58.0	79.1
19 F	0628	0956	1630	98.8	75.8	69.2	100	57.9	56.2	66.1	77.2	55.6	77.6

of conidia were trapped daily between the hours of 0530 and 1600 (Table 4) when leaf movement was absent or slight. *In situ* examination of conidiophores, with attached conidia, showed that conidial release was brought about by forcible discharge from the sterigmata (as recorded by Pinckard 1942). Its commencement appeared to coincide with the collapse of the base of the conidiophore where it emerged from the stoma. After the initial loss of turgidity of the conidiophore with the mycelium in the leaf, the collapse gradually extended upwards towards the superstructure bearing the conidia. Conidia were disseminated during this period but with further loss of turgidity in the stalk the whole structure collapsed and dissemination ceased. It was resumed on succeeding days and was not dependent on the occurrence of high humidity on the preceding night. The same daily cycle of dissemination occurred at humidities ranging from 40 to 90% but relatively few conidia were trapped at the higher humidities.

TABLE 5
TOTAL NUMBERS OF CONIDIA TRAPPED IN FIELD PLOTS

Numbers are for four one-hourly records commencing at 0830 hr, for 8 consecutive days

Day of March 1957	Total No. of Conidia	Day of February 1958	Total No. of Conidia	Day of February 1959	Total No. of Conidia
6	16	9	32	21	96
7	26	10	4400	22	16
8	250	11	3600	23	920
9	550	12	1340	24	700
10	4100	13	1991	25	266
11	2000	14	1685	26	262
12	200	15	1253	27	260
13	25	16	315	28	73

Sporulating leaves were dried and stored for several months then placed in a saturated atmosphere overnight. The leaves absorbed water and conidiophores became turgid. On subsequent redrying the conidia were discharged in the same manner as for fresh green leaves. Detached conidiophores on glass slides became turgid as a result of absorbing water through the stalk end rather than directly from the atmosphere. These observations support the view that turgidity of conidiophores is dependent on water supply from the leaf and not on ambient humidity. Thus conidiophores on green leaves would become turgid each night and discharge of conidia each morning would follow on the loss of turgidity due to development of a diffusion pressure deficit in the leaf.

(d) *Temperature and Humidity*

Under field conditions, temperature minima usually occurred at or soon after dawn (Fig. 1). As temperatures increased, the numbers of conidia trapped increased to a maximum at 3-4 hr before the time of maximum temperature. The increase in numbers commenced at temperatures of 56.2-59.1°F and maximum numbers were present at 66.1-70.9°F, but maximum temperatures recorded were 76.3-79.1°F

(Table 4). Thus a temperature rise of $9.9\text{--}11.9^{\circ}\text{F}$ during a period of 3.3–4 hr was associated with an increase in numbers of conidia disseminated. Under glass-house conditions the temperature rise was $5.8\text{--}13.1^{\circ}\text{F}$ during a period of 3–7 hr and the greatest numbers of conidia were trapped at temperatures near the maximum. Dissemination in the glass-house commenced at a higher temperature than in the field, then continued to a higher temperature and for a longer period (Table 4). In both field and glass-house, dissemination was associated with increase in temperature during the morning.

An increase in the number of conidia trapped in the field was associated with the decrease in humidity after sunrise but dissemination ceased before humidity reached its lowest point. Very few conidia were trapped during periods of near-saturation humidity during either day or night except when very large numbers were present on the leaves during the period of maximum production. Maximum numbers were trapped within the humidity range of 55–76%. Under glass-house conditions a drop in humidity was not always associated with an increase in conidial numbers whether or not that drop occurred in the morning or afternoon. In some glass-house tests, a maximum humidity at approximately 1400 hr was followed by a decline until 0900 hr on the next day. However, conidia were not trapped during this period but only during the normal daily dissemination period in the morning. In some tests conidial numbers increased during a period of rising humidity and temperature. Under near-saturation humidity an increase in temperature did not cause dissemination. Few conidia were trapped during periods of slow rates of change in temperature and humidity whereas rapid changes promoted dissemination. The summarized data in Table 4 show that dissemination is associated with an increase in temperature, that changes in humidity may be very small indeed, and that the daily pattern of dissemination occurs over a wide range of humidities.

(e) *Light*

The daily pattern in the numbers of conidia trapped was characterized by few to none during darkness, followed by an increase commencing soon after sunrise. In continuous darkness there was a gradual reduction in the number of conidia trapped per hour, and this trend was not changed by an increase of 5°F in temperature or a decrease of 6% in humidity. At humidities above 80% there was almost no dissemination. After either low or high humidity treatment for 4–5 days, during which very few conidia were trapped in darkness, the conidia could be disseminated by leaf movement and trapped in enormous numbers. In other experiments conducted in darkness, air temperature was increased by $2\text{--}18^{\circ}\text{F}$ and humidity decreased by 2–10%. Treatments commenced at 0900, 1000, 1100, 2000, or 2200 hr and were for periods of 2–8 hr. The tests were done within a range of 38–90% humidity and $62\text{--}88^{\circ}\text{F}$. There was relatively little effect of treatment on numbers of conidia trapped—a small increase during the 1–2 hr at the commencement and then a rapid decline to normal numbers. Similar results were obtained for each time of treatment. During this short period of increasing numbers, temperature and humidity varied slightly, much greater changes occurring during the period of decline in numbers.

The above results showed that continuous darkness was highly unfavourable for dissemination of conidia and further experiments showed a similar pattern for continuous light. In seven tests, using continuous light for 2–6 days, temperature ranges of 58–78°F, and humidities of 58–93%, there was a persistent decline in numbers of conidia disseminated with time, despite temperature and humidity changes of up to 8°F and 11% respectively. Heat treatments similar to those used in continuous darkness caused a small increase in numbers during the first 2 hr then a rapid decrease. There was no daily periodicity in numbers with continuous darkness or continuous light.

Light for periods of 5–30 min caused small increases, of the order of 2–6, in numbers of conidia trapped. With light for 4 hr the number increased from 5 to approximately 150 but with similar plants in the glass-house the increase was seven or more times as great. With light treatments of 8–12 hr, conidia were trapped for longer periods. Light treatments caused an immediate small increase in numbers then a decline followed by a further and sustained increase with continuous light for periods of up to approximately 9 hr. In other experiments under near-saturation conditions, neither light nor a temperature rise of up to 12°F caused dissemination.

When the effects of light and temperature treatments were compared it was found that increases in numbers due to increased temperature occurred for a short time at the beginning of the treatment and were followed by a rapid decline; whereas with light, an increased number of conidia were present for a maximum of approximately 9 hr. In the experiments with dark or light treatments or both conidia were disseminated as a response to light or temperature at any time of the day or night. Changes in conidial numbers were associated directly with the period of the treatment, whereas in the glass-house and field, increases and decreases in numbers were less sharply defined.

(f) *Viability of Conidia*

In 1957 viable conidia were trapped in the field throughout a 14-day period which included two peaks of dissemination. The highest germination rate recorded (50–60%) occurred in conidia trapped during the period 0900–1600 hr when the spore number in the atmosphere was at a maximum. Minimum and maximum temperatures for the 14-day period were 45 and 88°F respectively, the means being 55 and 77°F. Corresponding means under standard meteorological conditions (4 ft above soil level) were 52 and 80°F respectively. The relative humidity was above 95% for 5 or more hours on six of the 14 nights, but during the day was 40% or less for up to 4 hours on 8 days and between 40 and 60% on the remaining 6 days. Viable conidia were trapped throughout the full range of temperatures and humidities but not during periods of rising and high humidity at night except under special conditions as shown in Table 1. The highest germination recorded occurred in conidia trapped when the humidity was below 80%.

In the glass-house with the humidity continuously above 90% germination occurred in the conidia trapped from the same lot of plants throughout a period of 12 days, in another experiment for 8 days with humidities between 70 and 90%, for 5 days with humidities between 59 and 73%, and for shorter periods at lower

humidities. The highest percentage germination usually occurred in conidia disseminated from 6 to 30 hr after sporulation was first observed. Germination of conidia trapped in the glass-house was higher and more uniform than that occurring in conidia trapped in the field.

The data quoted above indicate that conidia survive low humidities and that humidity *per se* is not likely to be an important factor limiting the spread of viable conidia.

IV. DISCUSSION

The data reported in this paper show that conditions favouring dissemination of conidia differ from those required for their production. Thus Cruickshank (1958) listed threshold value requirements for maximum sporulation as 2.6 atm for diffusion pressure deficit in the leaf and 97% for relative humidity. Such high humidity did not favour dissemination but this could be brought about by violent leaf movement. The conidia were mature, as judged by their capacity for germination and, therefore could be considered as available for dissemination. Maximum numbers were present at 0800 hr (Cruickshank 1958); nevertheless, in these experiments very few were released into the atmosphere at that time. Even at much lower humidities few were airborne at 0800 hr and maximum numbers did not occur until 2 or more hr later. It was shown in further experiments that conidia could be produced under laboratory conditions at any time between 1800 and 0800 hr on the following morning, but spore-trap data did not indicate any increase in numbers that could be attributed to sporulation at any time other than 0500–0800 hr. In *Phytophthora infestans*, conidia can be produced throughout the day and night (Wallin 1953). Nevertheless, the daily cycle of dissemination is the same as that for *Peronospora tabacina*. From this it is concluded that conidia do not have to remain attached to the conidiophore for a particular period of time before dissemination can occur.

Yarwood (1956) concluded that, for downy mildews, high humidity was not essential during "the transfer of inoculum from its point of production to an infection court". The results obtained with *P. tabacina*, the causal fungus of downy mildew of tobacco, support this view. Greatest numbers of conidia were trapped under conditions of rising temperature, increasing insolation and decreasing humidity, all of which appear to be unfavourable for survival and the establishment of new centres of infection.

There were two patterns of dissemination. The more important, in terms of numbers of conidia and length of dissemination period, commenced soon after sunrise. The number trapped increased for 3–4 hr then decreased for a further 4–6 hr, peak numbers for all experiments being between 1000 and 1300 hr and for the field alone, between 1000 and 1100 hr. The second pattern was independent of time of day and due to particular treatments that could also be components of, but not major determinants of, the first pattern. It was characterized by fewer conidia and the increased numbers were present for only a few minutes. A direct association between treatments and conidial numbers trapped was established when it was found that conidia disappeared from the area of release within a few minutes of becoming airborne. The most important cause of this pattern was leaf movement.

Wind, rain, and mechanical shock cause leaf movement and a sharp increase in conidial numbers followed by an abrupt and almost immediate decrease. Few, if any, conidia were dispersed as a result of leaf movement during periods of near-saturation humidity nor did violent leaf movement prevent sporulation on plants held in a saturated atmosphere. In the field the number of conidia in the atmosphere increased sharply when leaves were disturbed by cultural operations or when gusts of wind, often associated with rain squalls or thunderstorms, caused leaf movement. Storms often occurred during the late afternoon when temperature and insolation were decreasing and the humidity increasing. Leaves remained wet for at least the 2-3 hr which other experiments have shown to be sufficient for germination (Angell and Hill 1932) even though atmospheric humidity for most of the time was well below saturation (Fig. 1, reference point 2). Such conditions favoured widespread dispersal of conidia, their germination, and subsequent infection of leaves. If similar conditions occurred during the morning very large numbers of conidia became airborne but if they occurred during the normal daily decline in numbers, the downward trend was interrupted, only to be followed by a second increase under conditions even more favourable for survival (Fig. 2, reference point 2). Observations in the field show that widespread infection is associated with wind and rain showers (Stover and Koch 1951) whereas the spread of infection to adjacent plants in both seed-bed and field can also occur during relatively calm periods such as are common in the mornings following sporulation associated with heavy dew.

Waggoner and Taylor (1958) reported a daily cycle of spore dissemination in *Peronospora tabacina* in Connecticut, U.S.A., and their results were similar to those previously obtained by Hirst (1953) for *Phytophthora infestans* in England. The daily cycle appears to have been accepted as following upon renewed sporulation during each night period. In the experiments reported above, diurnal periodicity persisted even after successive nights with humidity below 50% and therefore too low for sporulation. Diurnal periodicity did not occur in continuous darkness or continuous light except when atmospheric temperature was increased. With both temperature and light treatments conidia were trapped at whatever time of the day or night the treatments were imposed but the numbers were low in comparison with those trapped from similar lots of plants in the glass-house. From this it would appear that dissemination was a response to light or to an increase in temperature or to both factors. However, neither of these factors caused dissemination at humidities near saturation. A decrease in humidity was not essential for dissemination (Table 4), nevertheless it did not occur at atmospheric saturation. In the field, insolation, temperature, and humidity changed rapidly during the time that the number of airborne conidia increased to a maximum but the general trend of the evidence suggested that these factors were responsible for changes in the host plant and that such changes resulted in dissemination of conidia. In three widely separated parts of the world the peak in numbers of conidia becoming airborne occurred at approximately the same number of hours after sunrise. This supports the suggestion by Hirst (1958) that increased insolation was associated with spore release.

Neither Hirst (1953) nor Waggoner and Taylor (1958) present direct evidence on the role of humidity in dissemination of *Phytophthora infestans* and *Peronospora*

tabacina respectively, nevertheless their results showed that it was deferred by high humidity. They did not consider that temperature, wind, and rain were major factors but Hirst (1958) subsequently suggested that insolation may modify humidity at the leaf surface and that reduction in humidity would cause dissemination. Cruickshank (1958), using leaf disks, reported that conidia were discharged as ambient humidity was reduced but his results may have been a consequence of plant tissue response rather than a direct effect of the reduced humidity on the spore-bearing structures. In the glass-house experiments reported above decreases in humidity during the afternoon or night were often greater than those that occurred in the morning but there were no increases in numbers of conidia trapped. The normal morning period of dispersal could occur with or without any significant change in humidity and even if humidity was increasing. However, on the day of sporulation, the rate of increase in numbers of conidia in the atmosphere was greatest as humidity decreased from approximately 80 to 60%. At any humidity the number of conidia trapped in a calm atmosphere was relatively low compared with that in a turbulent atmosphere associated with varying humidity and temperature.

Under field conditions interaction of insolation, temperature, and humidity limited the usefulness of a statistical examination of the data, nevertheless for the one season analysed there was a marked trend towards association of rate of increase of temperature with conidial numbers but no evidence of an effect due to change in humidity.

As diurnal periodicity in release of conidia is maintained under a wide range of environmental conditions for several days (Hill 1960) there must be some mechanism for ensuring that all conidia are not dispersed on the day of production. The explanation for this phenomenon appears to be in the plant and pathogen association. In general, rate of transpiration in plants increases rapidly during the morning and attains a maximum by early afternoon while rate of uptake of water increases more slowly and attains a maximum in late afternoon. As the moisture content of the leaves decreases, the concentration of sap increases and there is a movement of water into the leaf (Kramer 1949). Factors influencing rate of transpiration were investigated by Kiuper and Bierhuizen (1958) who showed that leaf temperature, air temperature, and relative humidity determine the value of the vapour pressure deficit. Leaf temperature is influenced by light, and transpiration follows solar radiation. Kajiwarra and Iwata (1959) examined the diurnal cycle of sporulation and the effect of light on *Pseudoperonospora cubensis* (Berk. & Curt.) Rostov. and suggested "that alternating light and darkness may be responsible for cyclic manifestation of sporulability in the cucumber downy mildew, by indirect effect through the host plant".

Production of conidiophores is dependent on a low diffusion pressure deficit (Cruickshank 1958), therefore it could be expected that, after production, they would be responsive to an increase in the diffusion pressure deficit. Transpiration from diseased leaf tissue is greater than from healthy tissue (Cruickshank and Rider 1961) and this could be a factor in the collapse of the conidiophores commencing adjacent to the leaf epidermis. Some conidia are dispersed during the period of collapse and the remainder become available for dispersal when the cycle is repeated.

On this hypothesis, diurnal periodicity would not be dependent on the occurrence of a high night humidity followed by a low morning humidity. Changes in light, temperature, and humidity would be of importance in their effect on the diffusion pressure deficit of the leaf, relatively rapid changes being followed by dispersal of conidia for short periods. Previous investigators have regarded the conidiophore as a self-contained unit directly responsive to ambient humidity and acting independently of the host plant whereas the results reported here suggest that conidiophore response is conditioned by changes in the host plant. The net result of this mechanism is that a pathogen with limited opportunities for sporulation can disseminate conidia at any favourable opportunity over a period of days with consequent improvement in prospects for infection and spread of disease.

As conidia were disseminated from both living and dead leaves their release from the conidiophore could not be accepted as evidence of viability. The highest percentage germination was obtained with conidia trapped within almost a day of their production; nevertheless, in both field and glass-house experiments some germination occurred in conidia trapped days or weeks later. In general, germination occurred in conidia trapped from green plants during the daily cycle and was observed during the days over which the cycle persisted. This indicates that conidia tend to retain viability for several days after production despite low atmospheric humidity, a conclusion that is supported by tests on glass slides in the laboratory (Angell and Hill 1931).

The atmospheric conditions during the morning of production are of particular importance in relation to dissemination in the field. If, as usually happens after a heavy dew, the atmosphere remains relatively calm, a large number of conidia would be disseminated over a very limited area (Schrodter 1954; Ullrich 1958). The area would be greater when the plants are small than when they are large (Hirst 1958) and, in tobacco plants nearing the flowering stage, may include only those plants adjacent to the sporulating plant. Thus dissemination during periods of increasing insolation and temperature, and decreasing humidity, which are characteristic of the diurnal cycle, ensures only a localized increase in the number of disease lesions. This has been observed repeatedly in the field and is often referred to by growers as "the slow spread of spot mould".

Conidia produced during wet, windy weather or subject to such weather would be disseminated over a wide area (Schrodter 1954). Such conditions could occur during the normal morning dissemination period, when the number of conidia available is very high, or at other times of the day (Figs. 1 and 2). In the tests reported here atmospheric humidity as a result of rain during the day was rarely high enough to prevent dissemination, and leaves remained wet for at least the 2-3 hr necessary for establishment of infection.

When dissemination is considered in relation to disease control it appears that conidia from small field plants have a much greater potential for widespread distribution than those from large plants. Accordingly, the most effective control in the field would be obtained by the prevention of infection and sporulation in small plants. If the disease occurs, control sprays applied before the time of the daily dissemination period could be expected to give better results than those applied later. In framed

seed-beds, crowded seedlings and relatively wind-free conditions tend to limit atmospheric dissemination of conidia but mechanical dissemination during weeding and removal of seedlings for transplanting would be of particular importance.

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STUDIES CONCERNING THE INHERITANCE OF ASCOSPORE LENGTH IN *NEUROSPORA CRASSA*

I. STUDIES ON LARGE-SPORED STRAINS

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Summary

Previous work has shown that the inheritance of ascospore size in certain strains of *Neurospora crassa* is controlled by a polygenic system with a small cytoplasmic component. Various types of selection lines were set up in strains that had been selected for the large-spored character. The results of these selection procedures are discussed. Experimental work which gives a better estimate of the linkage of part of this polygenic system to the albino locus on chromosome I is reported.

I. INTRODUCTION

It has been shown (Pateman 1955, 1959) that the length of ascospores in *Neurospora crassa* is largely controlled by a polygenic system with a small cytoplasmic component. In this work (cf. Pateman 1959), simple selection for increased ascospore length was practised. The mean ascospore length increased from about 18 units in wild-type crosses to a mean ascospore length of about 23 units after eight generations of selection. Further simple selection for increased ascospore length failed to increase the mean ascospore length over a further eight generations. Additional selection experiments with these strains and other studies on the inheritance of ascospore length in *N. crassa* are described in this paper.

II. MATERIALS AND METHODS

The strains used were 232-1, 232-2, 232-3, and 232-4, all of which were albino (*al* 2), large-spored strains obtained from a single ascus at the fifteenth generation of selection for increased ascospore length. The wild-types, St. Lawrence A (St.L.A) and 1196*a*, were originally derived from the Abbot or Lindegren wild-types or both and are largely isogenic, since 1196*a* was derived from seven generations of backcrossing to St.L.A (Pateman 1959). The vegetative cultures were maintained on Fries No. 3 (Beadle and Tatum 1945) medium. All crosses were made on agar slopes of a medium favouring sexual reproduction (Westergaard and Mitchell 1947) and normally incubated at 25°C.

The length of the ascospores was measured using a projection microscope and the unit of measurement was approximately 1.73 μ .

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III. EXPERIMENTAL RESULTS

(a) Selection Lines Derived from the Large-spored Genotype

In earlier selection experiments (Pateman 1959) a response to selection was obtained over the first eight generations and although selection for increased ascospore size was practised for a further eight generations no further response was obtained. However, this response to selection was accompanied by a decrease in fertility in the selected lines. Strains from the fifteenth generation of selection for increased ascospore size were taken as a starting point for further selection in order to find out if any genetic variability still existed in them. The following series of selection lines were set up.

(i) *The High Line*.—The procedure was as follows. Crosses were set up between 232-1 and 232-3, and 232-2 and 232-4. From these crosses phenotypically large ascospores were isolated and the strains thus obtained test-crossed to the large-spored parents. A sample of 50 ascospores was measured from each cross. The strains that gave the highest mean ascospore size in the test crosses were then crossed. Two crosses in which each of the four parents were dissimilar were used for the selection of the next generation. This procedure was used in all succeeding generations.

This form of selection was adopted for the following reasons. First, simple selection had been practised for a prior eight generations without increasing the mean ascospore size. Second, there is more chance of genetic variability showing up in this type of performance test, than in simple selection for ascospore length. Third, the course of selection implies a selection for fertility.

The results of this selection are illustrated in Figure 1. There was an advance of about 2 units over the level of the original selected line. Thus the total response to selection for increased ascospore size is about 9 units of which about 7 units net increase was obtained by Pateman (1959). Crosses between strains in this selection line were very much more fertile than crosses between the strains from which they originated. The regression line through the values of this high line is significantly different ($t_7 = 6.44$; $P < 0.001$) from a line of zero slope at the mean value of 14 crosses between the originating strains. This suggests that there was some genetic variability in the original strains even after 15 generations of selection and even though there had been no response for the last eight generations of this selection.

(ii) *The Low Line*.—In this case a simple selection was practised. In each generation the shortest ascospores were selected and the strains so obtained were intercrossed. From these crosses two fertile crosses in which each of the four parents were dissimilar were selected to carry on the next generation. This procedure was used in all succeeding generations.

After 12 generations, if there was any response, it was a slight but non-significant increase in mean ascospore size (Fig. 1). However, the decrease in fertility was very rapid and after a few generations it was difficult to obtain enough ascospores to measure and germinate. This decrease in fertility became evident after

two generations and in order to try to restore the fertility, mass culture lines were started at generations 2 and 6. In these lines the fertility improved slightly and the mean ascospore size decreased, but the decrease was not significant.

These results suggested that the amount of genetic variability left in these strains was not sufficient to allow any decrease in ascospore size using this form of selection. Also when selection was relaxed as in the mass culture lines, the strains show a marked stability about the level obtained by Pateman (1959).

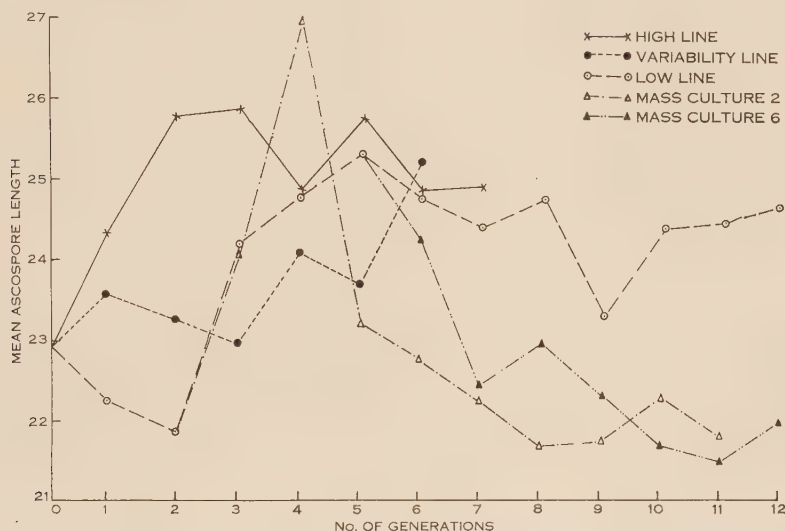


Fig. 1.—Effect of selection on large-spored strains of *Neurospora crassa*.

(iii) *The Variability Line*.—In each generation the shortest and longest ascospores were isolated and the strains so obtained were crossed together, so that a strain derived from a short ascospore was always crossed to a strain derived from a long ascospore. Two crosses in which all four parents were dissimilar were used to carry on the next generation. This procedure was used in all succeeding generations.

As can be seen (Fig. 1) a slight but significant increase ($t_6 = 2.6$; $P < 0.05$) in mean ascospore size has occurred in six generations.

The results indicate that there was some genetic variability left in these strains after 15 generations of selection for increased ascospore length. This was sufficient for an increase in mean ascospore length in two of the lines, but not sufficient for a decrease in mean ascospore length. In all selected lines there has been an increase in mean ascospore length. This indicates that, in fact, selection for deviants from the mean ascospore length, i.e. selection for variability of ascospore length, is important in this genetic system.

In the original selection for increased ascospore length, the coefficient of variation had increased from 5 to 17% by the time the line had plateaued (Pateman

1959). In all these selection lines the coefficient of variation remained at or near this value. This indicates that no further instability in ascospore or ascus development over that found by Pateman (1959) has become evident.

In all selection lines, except the high line, the response to selection was accompanied by a decrease in fertility. This was due to three main causes:

- (1) Fewer perithecia were formed in crosses between selected strains than in crosses between the original strains.
- (2) Of the ascospores produced in crosses between selected strains about 25% did not mature.
- (3) Of the mature ascospores produced in crosses between selected strains about 25% did not germinate.

Even in the mass culture lines, where selection for ascospore length was relaxed and selection for fertility practised, the fertility returned to the level of the originating strains only. Thus it seems likely that the genotype has stabilized around the value of the mean ascospore size of the originating strains.

(b) Linkage Experiments

Previous work (Lee and Pateman 1959) indicated that a part of the polygenic system responsible for approximately one-sixth of the total response to selection over 15 generations was apparently linked to the albino (*al* 2) locus on chromosome I which also contains the mating-type locus (*A*, *a*). The initial estimate of the degree of linkage was 9 ± 6 centimorgans (cM). In order to try to locate this genotypic material more accurately, a further series of experiments was undertaken.

The strains 232-1, 232-2, 232-3, and 232-4 were crossed to the appropriate wild type and the crosses incubated at 25°C. The F_1 ascospores were normal in size. One hundred ascospores were randomly isolated from each cross. The isolates thus obtained were each backcrossed to the large-spored parent. This gave a total of 73 crosses in which albino F_1 strains were backcrossed and 102 crosses in which non-albino F_1 strains were backcrossed. A sample of 50 ascospores from each cross was measured. The results are presented in Table 1. The majority of backcrosses involving albino F_1 strains had mean ascospore lengths in the range 19.42–22.88 units, while the majority of backcrosses involving non-albino F_1 strains had mean ascospore lengths in the range 17.26–18.94 units. The difference, 1.18 units, between the two groups of backcrosses is highly significant ($P < 0.001$). This indicates that some of the genotypic material controlling ascospore length is linked with the albino locus.

From Table 1, it can be seen that 10 out of 73 albino F_1 strains when backcrossed gave a mean ascospore length in the range 17.26–18.94 units and 13 out of 102 non-albino F_1 strains when backcrossed gave a mean ascospore length in the range 19.42–22.88 units. It is reasonable to regard these exceptions as due to crossing-over between the albino locus and the site of the genotypic material controlling this part of the response to selection for increased ascospore size. If this is so, then this genotypic material is located about 13 ± 5 cM from the albino locus, calculated on the basis of 23 recombinants in a total of 175 backcrosses.

The ascospore length in the majority of backcrosses fell into one or other of two distinct classes. There were, however, two albino F_1 strains where the mean ascospore length was intermediate between the two classes. These two intermediate cases are probably due to the segregation of the genotypic material controlling the rest of the response to selection for increased ascospore size. These intermediate cases have not been included in the estimation of the degree of linkage of the genotypic material to the albino locus. Attempts to locate this genotypic material using other biochemical markers have not yet been successful. So the following method of establishing the exact location is the best available at the moment.

TABLE 1
BACKCROSSES OF *al* AND al^+ F_1 STRAINS TO LARGE-SPORED PARENTS
1 unit = 1.73μ . For difference between means $t_{175} = 7.5$; $P < 0.001$

	<i>al</i> F_1	al^+ F_1
No. of backcrosses with mean in the range 17.26–18.94 units	10	89
No. of backcrosses with mean in the range 19.42–22.88 units	61	13
No. of intermediate backcrosses	2	0
Total number of backcrosses	73	102
Mean ascospore length (units)	19.68	18.50

Considering the cross $232-1 \times 1196a$, the genotypic material linked to the albino locus was called *L* and in backcrosses to the large-spored parent gave a mean ascospore size in the range 19.42–22.88 units. If *L* is assumed to be proximal then the cross can be represented as $A-L-al \times a-L^+-al^+$. The region between *A* and *L* was called region I, while the region between *L* and *al* was called region II. Then, in this cross when only strains of mating-type *a* were backcrossed to the large-spored parent, the following genotypes resulted and represent the following cross-over types: $a-L^+-al^+$, parental, $a-L-al^+$ crossover in regions I and II, $a-L^+-al$ crossover in region II, and $a-L-al$ crossover in region I. Applying this argument to each of the other sets of backcrosses, when *L* is assumed to be proximal to *al*, the genotypes and crossover classes are as presented as in Table 2.

Again, if *L* is assumed to be distal to *al*, then the cross $232-1 \times 1196a$ may be represented as $A-al-L \times a-al^+-L^+$. The region between *A* and *al* was called region III and the region between *L* and *al* was called region II. Then, when strains of mating-type *a* only were backcrossed to the large-spored parent, the following genotypes resulted and represent the following crossover types: $a-al^+-L^+$, parental, $a-al^+-L$ crossover in region II, $a-al-L^+$ crossover in regions II and III, and $a-al-L$

crossover in region III. Applying this argument to each of the other sets of backcrosses, if *L* is assumed to be distal to the *al* locus, the genotypes and crossover classes may be tabulated as in Table 3.

TABLE 2
GENOTYPES AND CROSSOVER CLASSES WHEN *L* IS ASSUMED TO BE
PROXIMAL TO *al*

Genotype	Region where Crossover Occurred	No. of Cases
$\left. \begin{array}{l} A-L^{+}-al^{+} \\ a-L^{+}-al^{+} \end{array} \right\}$	None	89
$\left. \begin{array}{l} A-L-al^{+} \\ a-L-al^{+} \end{array} \right\}$	I and II	13
$\left. \begin{array}{l} A-L^{+}-al \\ a-L^{+}-al \end{array} \right\}$	II	10
$\left. \begin{array}{l} A-L-al \\ a-L-al \end{array} \right\}$	I	61

In the genotypes represented in Tables 2 and 3, all strains of mating-type *a* were backcrossed to either 232-1 or 232-2 and all strains of mating type *A* to either 232-2 or 232-4.

TABLE 3
GENOTYPE AND CROSSOVER CLASSES WHEN *L* IS ASSUMED TO BE
DISTAL TO *al*

Genotype	Region where Crossover Occurred	No. of Cases
$\left. \begin{array}{l} A-al^{+}-L^{+} \\ a-al^{+}-L^{+} \end{array} \right\}$	None	89
$\left. \begin{array}{l} A-al^{+}-L \\ a-al^{+}-L \end{array} \right\}$	II	13
$\left. \begin{array}{l} A-al-L^{+} \\ a-al-L^{+} \end{array} \right\}$	II and III	10
$\left. \begin{array}{l} A-al-L \\ a-al-L \end{array} \right\}$	III	61

When *L* is assumed proximal to the albino locus, the map distances are *A* to *L* 42 cM and *L* to *al* 13 cM. Now the map distance between *A* and *al* is known to

be 38 cM (Barratt *et al.* 1954), so assuming this value, the expected number of double crossovers between *al* and *A* would be 3.2%. In fact that actual value is 7.5%. Again, the distance between *A* and *al* calculated on the assumption that *L* is proximal to *al* is 55 cM, which does not correspond with the known value of 38 cM. This cannot be due to any chromosomal abnormalities since the same data when *L* is assumed to be distal to *al* gives a very reasonable value of 41 cM.

If, on the other hand, *L* is assumed to be distal to the albino locus, the map distances are *A* to *al* 41 cM, and *al* to *L* 13 cM. So assuming the distance between *al* and *A* is 38 cM, the expected number of double crossovers would be 5.0% and in fact the value obtained from the data is 5.8%. Also, the calculated distance between *al* and *A*, 41 cM, corresponds quite well with the known value of 38 cM. So the assumption that *L* is distal to the albino locus is much more likely than the assumption that *L* is proximal.

IV. DISCUSSION

In the selection lines derived from the large-spored genotype, only the high line and variability line showed any significant response over the level of ascospore size obtained by Pateman (1959). Although environmental variation has a large effect on these large-spored strains, there is still some genetic variability left, but not enough to respond to simple selection, either for large or small ascospores.

In the low and variability lines developmental instability occurred as a result of selection. This was reflected in a decrease in the number of ascospores produced, an increase in the number of aborted ascospores and asci, and a decrease in the number of mature perithecia produced. This decrease in fertility is similar to that obtained by selecting for differences in bristle number in *Drosophila melanogaster* (Mather and Wigan 1942; Harrison and Mather 1950) and can probably be regarded as a correlated response. The mass culture lines set up to try to improve the fertility of the low line gave an increase in fertility, yet no significant drop in mean ascospore length. This indicates that the selection for ascospore size practised by Pateman (1959) has been effective in increasing the mean ascospore length for these strains to about 23 units and stabilizing the genotype at this level. This is further substantiated by the fact that no further changes in the coefficient of variation over that found by Pateman (1959) occurred in these selection lines even though further developmental instability resulted.

The establishment of a linkage relation between a part of this polygenic system and the albino (*al* 2) locus on chromosome I has been previously reported (Lee and Pateman 1959). This part of the polygenic system is responsible for about one-sixth of the total response to selection for large ascospore size over 15 generations. This cannot be due to pleiotropic effects of the albino locus because recombination between the polygenic material and the albino locus has been demonstrated.

Apparent linkage between polygenes and major genes has been shown in *Phaseolus vulgaris* (Sax 1923) between seed size, a polygenic character, and the colour gene *P*. This may have been due, as Haskell (1959) suggests, to pleiotropic effects of this colour gene. However, the linkage shown in *Pisum* (Rasmusson 1935) between polygenes controlling flowering time and a major gene governing pi men-

tation is free from this objection since recombination between the polygenes and the major gene locus has been shown. Again in *D. melanogaster* many cases are known of the location of areas of polygenic activity. Mostly these cases are concerned with the location of polygenes controlling bristle number (Mather 1941, 1942, 1944; Wigan 1949). So far, additional work with other markers has failed to locate this genotypic material with greater accuracy. This is probably because of interaction between the marker alleles used and the polygenic material. If this material can be located and bounded by marker alleles which do not interfere with its phenotypic expression, then it may be possible to make a direct study of recombination within a group of polygenes which exhibit linkage in a crude analysis.

V. ACKNOWLEDGMENTS

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BIOELECTRIC OSCILLATIONS OF BEAN ROOTS: FURTHER EVIDENCE FOR A FEEDBACK OSCILLATOR

I. EXTRACELLULAR RESPONSE TO OSCILLATIONS IN OSMOTIC PRESSURE AND AUXIN

By I. S. JENKINSON* and B. I. H. SCOTT†

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Summary

Bean roots subjected to oscillations in osmotic pressure or in the auxin concentration of their weakly conducting bathing medium exhibit corresponding oscillations in their bioelectric fields with a resonance at the natural period of oscillation (approx. 5 min). The amplitude and phase responses, expressed as a function of the periods of the applied oscillations, are in agreement with those predicted from a theoretical model of a feedback loop. A physiological model compatible with the theoretical one is suggested.

I. INTRODUCTION

In a previous paper from this Laboratory, Scott (1957) described spontaneous oscillations in bioelectric potentials produced by bean roots maintained in an unchanging environment. It was suggested that these oscillations could be caused by a closed-loop feedback system of control acting between certain physiological variables. The suggested variables were the electric field, the auxin supply, and membrane permeability. In the present paper experiments which were devised to test this hypothesis are described.

One of the standard methods of investigating a feedback system is to apply an external oscillation of varying frequency to one of the variables of the feedback loop. From the resulting amplitude and phase responses of all the variables, the properties of the loop may be determined. Alternatively, if only one of the variables in the loop can be measured the properties of the loop may be found from its response to oscillations applied separately to each of the other variables in the loop. In the case of the bean root system under investigation, oscillations of other elements in the proposed loop besides the electric field have not been observed. Attempts to measure resistance changes corresponding to the postulated permeability oscillations were successful. Consequently only the second of the methods of investigation mentioned above was feasible.

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If the bioelectric field forms part of a feedback loop it would be expected to show characteristic responses when an oscillatory electric field is applied to the plant root. Preliminary experiments were performed in order to look for this response but it was found that there were considerable practical difficulties in separating the applied oscillatory potentials from any resulting bioelectric oscillations, and the experiments were discontinued.

Two experimental treatments have been found which result in oscillation of the bioelectric potential. In the first of these the osmotic pressure of the root's bathing solution is changed rhythmically. The oscillation in the bioelectric potential probably results from an oscillation in the water content and hence salt concentration of the outer cells of the root. In the second treatment an oscillatory concentration of β -indolylacetic acid (IAA) is applied to the root's bathing solution.

In this paper the results of experiments using these treatments are described and discussed in terms of a simple feedback loop oscillator.

II. EXPERIMENTAL MATERIAL AND METHODS

Broad beans (*Vicia faba* L. cv. Long Pod) were grown at 25°C in tap water which was continuously circulated and aerated. Two-day-old plants with roots about 3 cm long were used.

In most experiments the plants were removed from the culture bath and set up in the bathing solution of the measuring tank only about an hour before the commencement of the experiment. This time would probably not allow for complete equilibration of the plant roots with the bathing solution. However, in some series of experiments, the plants were allowed to equilibrate in the bathing solution for 15 hr or more before measurements were begun. In most experiments 10^{-4}M KCl was used as the bathing solution but in some cases 10^{-4}M CaCl_2 was used. The bath temperature was 25°C.

Bioelectric potentials were recorded automatically at five points in the bathing solution close to the plant root, using a six-channel recorder described by Scott (1957). The growth meter also described by Scott (1957) was used in some experiments to record the rate of elongation of the root. A recording chart speed of 3 in./hr was used throughout.

In order to produce an oscillation in osmotic pressure of the plant's bathing solution without changing the environment in any other way, a soluble unionized substance to which the plant membranes are practically impermeable is required. The substance must be unionized otherwise the conductivity of the plant's bathing solution and hence the bioelectric potentials would be affected. Sucrose has been used in most of the osmotic pressure experiments described in this paper. Although plant membranes have only a very low permeability to sucrose, it is, however, a physiologically active substance even in small concentrations once it enters a tissue (Brown and Sutcliffe 1950). Consequently the osmotic pressure experiments were repeated with mannitol which has the advantage of being largely physiologically inactive as well as being almost as non-permeating as sucrose. The results obtained with mannitol and with sucrose were in agreement.

The IAA used in these experiments was weighed out 10 mg at a time and then dissolved in 10 ml of absolute alcohol. This was stored away from light and was made up with 10^{-10}M KCl to the required concentration on the day of use. While in use the stock bottle containing the IAA in aqueous solution was shielded from light to help prevent deterioration. The concentrations of IAA in aqueous solution used in these experiments were 10^{-9} , 10^{-7} , and 10^{-5}M , respectively.

Figure 1 shows a diagram of the apparatus used to provide an oscillatory concentration of sucrose (or IAA) in a solution of constant KCl concentration (10^{-4}M). Two constant-head supply bottles, one containing 10^{-4}M KCl and $\text{M}/30$ sucrose, and the other containing only 10^{-4}M KCl, feed the two containers shown on the left of the diagram. The supply is adjusted so that the levels in each container are at the same constant height throughout. One end of each syphon tube dips into each container so that the rates of liquid supply to the feed tubes are determined by the heights of the syphon-tube outlets. These in turn are determined by the positions of the two eccentric circular cams on which the rigid arms supporting the syphon tubes rest. One end of each syphon tube dips into each container so that the rates of liquid supply to the feed tubes are determined by the heights of the syphon-tube outlets. These in turn are determined by the positions of the two eccentric circular cams on which the rigid arms supporting the syphon tubes rest.

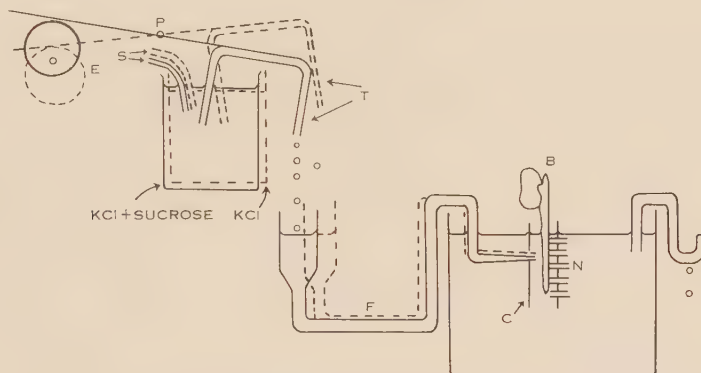


Fig. 1.—Schematic diagram of apparatus used to produce oscillations in osmotic pressure (or IAA concentration) in the bathing solution of plant root. The rear supply path is shown by the dotted lines. *E*, eccentric cams; *P*, fixed pivot; *S*, supply from reservoir bottles; *T*, syphon tubes; *F*, feed tubes; *B*, bean plant; *C*, polystyrene cylinder; *N*, "Nylex" tubes.

The cams are held on a single shaft so that they cannot rotate relative to one another. Further, the cams are opposed so that when one syphon tube is at its greatest height the other is at its lowest. In this way a maximum supply rate of sucrose plus KCl solution is delivered to one feed tube while a minimum supply of pure KCl solution is delivered to the other. When the camshaft is rotated by a half revolution the pure KCl solution supply rate is a maximum while the sucrose plus KCl solution supply rate is a minimum. The solutions are delivered via the feed tubes syphoning into the small polystyrene cylinder containing the bean root, the total rate of supply of solution being approximately constant throughout the cycle. The potential measuring probes placed at various distances along the plant root are held in holes drilled through the cylindrical container.

The camshaft is driven so that it advances by one-hundredth of a revolution every time an electric impulse is applied to the drive relay. The electric impulses are produced by an electronic pulse generator, the pulse repetition frequency of which can be adjusted continuously from 2 to 60/min. In this way the period of rotation of the shaft and consequently the period of the sucrose (IAA) concentration oscillation may be varied from about 1.7 to 50 min. However, the periods used were normally in the range 2–12 min.

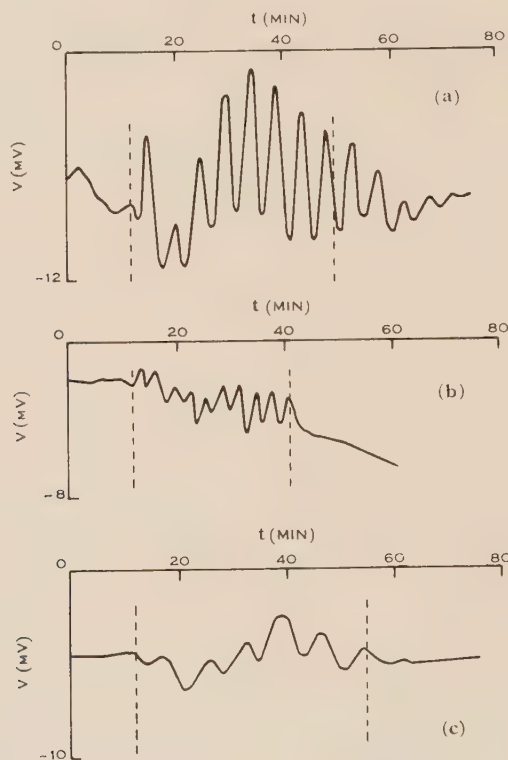


Fig. 2.—Potential response to osmotic pressure oscillations (0–M/30 sucrose) of three periods: (a) 4.7 min (the resonant period, T_r); (b) 3.0 min; (c) 7.5 min. The oscillation is applied between the dotted lines.

The phase of the applied oscillation in concentration (sucrose or IAA) is recorded on the potential record chart by automatically applying a voltage sufficient to deflect the pen to an off-scale position when the supply rate of sucrose plus KCl solution becomes a maximum. Since the total solution supply rate remains approximately constant (20 ml/min) throughout the cycle and the volume of the cylinder surrounding the plant is only about 1 ml, there is only a negligible phase difference (a few degrees) between the sucrose concentration round the plant and the supply rate of sucrose plus KCl solution even for periods as short as 2 min. Further, it

may be shown that the concentration maxima and minima deviate from the nominal values ($M/30$ and 0 for sucrose; $10^{-9}M$ and 0, etc. for IAA) by less than 1% even for the 2-min period. The delay involved in the syphon tubes is estimated to be negligible (about 1 sec).

III. RESULTS

(a) *Osmotic Pressure Response*

When the osmotic pressure of the plant root's bathing solution is oscillated, the bioelectric potential is forced to oscillate with the same period as the applied osmotic pressure oscillation. Figure 2(a) shows the potential response to the application of an osmotic pressure oscillation, the period of which is close to the natural period of oscillation for the plant. The natural period is the period of the transient oscillations which are generally observed when the plant is set up for measurement or its environment is disturbed (Scott 1957; Jenkinson 1958.) After removing the osmotic pressure oscillation, the potential oscillation is seen to continue for a number of cycles though it is damped. Moreover, the amplitude response is much greater than that in Figures 2(b) and 2(c) where the period of the applied osmotic pressure is considerably different from that of the plant's natural potential oscillation. In such cases (Figs. 2(b), 2(c)), the potential oscillation disappears as soon as the applied oscillation is removed.

For a particular period the amplitude of the potential response depends on the amplitude of the applied osmotic pressure oscillation. It has been found that for oscillations in sucrose concentration with peak values lower than $10^{-3}M$ the extracellular potential response is negligible. Between $10^{-3}M$ and $3 \times 10^{-2}M$ the potential response increases with molarity and above $3 \times 10^{-2}M$ it increases only slightly up to the plasmolysis threshold. Since the potential response does not increase appreciably beyond $M/30$ and since this molarity is well below the plasmolysis threshold, it has been chosen to give the maximum osmotic pressure in most experiments. This maximum pressure is about 0.8 atm.

In Figure 3 the potential responses to five different applied periods of osmotic pressure oscillation are shown. These potentials were recorded simultaneously at three different regions along the root. At all three positions a potential oscillation is produced, its period being equal to that of the applied osmotic pressure oscillation. The oscillatory response is again greater for periods near the natural period of the root (about 5 min), this being particularly noticeable in the potential recorded at the elongating region. Further evidence for a close correlation between the period at which the plant's response to an applied oscillation is greatest (i.e. the resonant period) and the natural period of the plant is given in Figure 4(a) where the values obtained for a number of plants are compared.

The response to applied oscillations with resonance near the natural period is generally found to be more marked in the elongating region than in other regions such as the root tip, primary meristem, or root base. Further, the oscillatory potentials at these regions are substantially in antiphase with those produced at the actively resonant, elongating region. It appears that these other regions are

not so actively resonant and that their potential oscillations are of a passive type caused largely by return currents produced in the actively resonant (elongating) region.

By means of the following experiments it has been possible to test the hypothesis that the actively resonant region is located in the elongating zone of the root (between 2 and 12 mm in the bean roots used), and not in the primary meristem nor in the regions where cell elongation has ceased. First the osmotic pressure oscillation

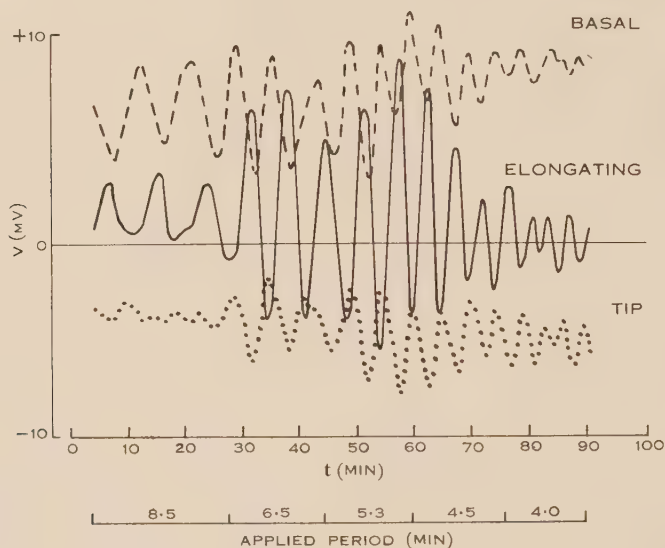


Fig. 3.—Potential response to osmotic pressure oscillations (0-M/30 sucrose) at three regions of the root. The period of the oscillation is reduced in steps from 8.5 to 4.0 min. Note the phase difference between the oscillation at the elongating region and at other parts of the root.

was set at the resonant (or natural) period. This caused the plant to produce enhanced oscillations at the same period. Three millimetres of tissue from the tip end of the root were then cut away thus removing the primary meristem. This treatment did not affect the response of the plant to the resonant period in any way, within 1 or 2 hr of the excision. However, the removal of a further 10 mm greatly inhibited the response to the resonant period, and in some cases completely suppressed the plant's potential response to the osmotic pressure oscillation.

In some experiments the resonant conditions was first evoked with most of the root (c. 3 cm) immersed in the bathing solution. Then the plant was raised so that only the last 10 cm remained in the bathing solution. The resonant oscillations continued although the background potential pattern (i.e. the steady potential pattern on which the oscillations are superimposed) was diminished. Excision of the first 3 mm from the tip end again did not diminish the oscillatory potential response.

This type of experiment was repeated using plants with roots only above 15 mm in length. In such roots there is hardly any tissue which has ceased elongating. These plants again exhibited strong resonances which were not inhibited by excising the primary meristem. These experiments show convincingly that the elongating zone of the root is the actively resonant region.

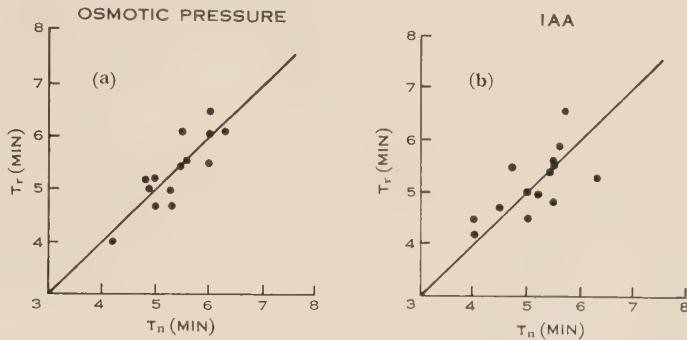


Fig. 4.—Relation between the resonant period (T_r) and the natural period (T_n) for a number of plants used in the osmotic pressure and auxin experiments.

(b) IAA Response

Resonant responses, very similar to those evoked by oscillations in osmotic pressure, have been observed when the concentration of IAA in the bathing solution is oscillated between 0 and $10^{-7}M$. The period of resonance again shows good agreement with the plant's natural period (Fig. 4(b)). If $10^{-9}M$ IAA is used as the peak concentration in the cycle, resonance still occurs but the response to all periods of oscillation is usually diminished considerably. The response to oscillations in IAA with $10^{-5}M$ peak concentration is small and no resonance is observed. This is probably because at such high concentrations the elongation of the root is completely inhibited (Scott, McAulay, and Jeyes 1955). Since oscillations between 0 and $10^{-7}M$ IAA evoke the most marked effect, most of the results for IAA in this paper refer to this peak concentration.

For the bioelectric oscillations evoked by IAA oscillations, the actively resonant region is again the zone of elongating cells in the root. This was shown in the same manner as for osmotic oscillations by excising different parts of the root.

Routine observations of growth rate using the growth meter described by Scott (1957) have shown that in certain isolated instances there are irregular oscillations in the rate of elongation of the same period as the resonant potential oscillations evoked by osmotic pressure or IAA. However, it does not appear that potential oscillations at resonance show any general correlation with oscillations in the overall rate of elongation of the root.

(c) Dependence of Amplitude and Phase of Bioelectric Oscillation on Period

Figure 5 shows typical examples of the dependence on period of the amplitude and phase (defined below) of the electric response in the active region for roots subjected to oscillation in osmotic pressure and IAA concentration. The natural periods of oscillation, obtained from transient potential data for each of the plants involved, are shown on the period (T) axis. It is seen that the resonant period is in agreement with the natural oscillatory period for each plant, both for osmotic pressure and IAA.

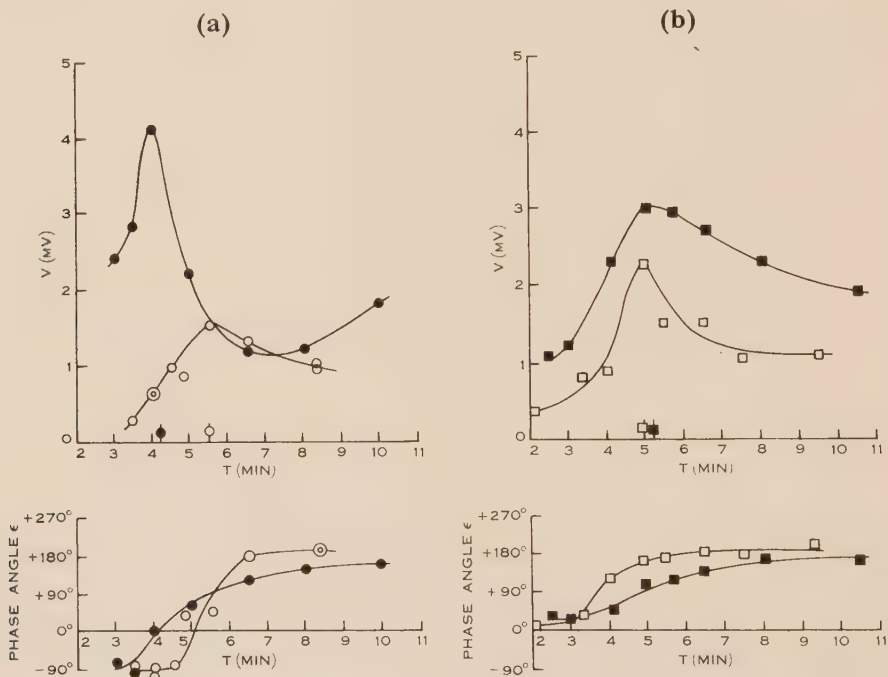


Fig. 5.—Amplitude and phase responses in the elongating region for (a) two plants subjected to osmotic oscillation (0-M/30 sucrose) and (b) two plants subjected to auxin oscillation (0-10-7M IAA). V is the double amplitude and ϵ the phase (defined in text) of the electric oscillation for applied oscillations of period T . The natural periods are indicated on the horizontal axes.

The phase angles shown in Figures 5(a) and 5(b) for osmotic pressure and IAA are defined as follows: If the algebraic maximum of potential precedes the maximum concentration of sucrose (or IAA), the potential leads, and the phase lead is shown as a positive phase angle. If the potential lags, the phase angle is negative. It is seen that the phase angle changes as the period is increased, the greatest rate of phase change being near the resonant period. At long periods, the observed potential oscillation is 180° out of phase with the applied oscillation in osmotic pressure or IAA concentration. However, the change in phase angle from short to long periods is greater for osmotic pressure than for IAA. For osmotic pressure the change is about 270° while for IAA it is only about 180°.

In Figure 3 it was seen that the phase of the forced potential oscillations is not the same for all points along the root, the oscillations produced at the elongating region being substantially, though not exactly, in antiphase with those observed at the more passive regions. Hence, if phase response curves such as those in Figure 5(b) were drawn for the potential responses at the passive regions, these curves would be displaced by about 180° with respect to those in Figure 5(b) for the elongating region. The phase curves for the passive regions, however, still show the same phase change (about 270° for osmotic pressure) from short to long period. In some cases even the phase curves for the elongating regions of different roots

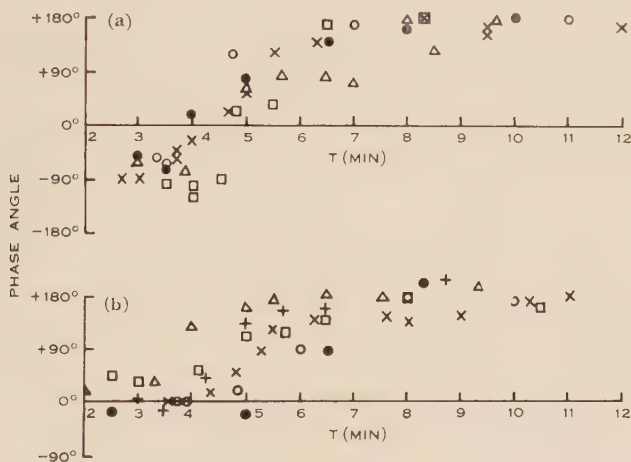


Fig. 6.—Normalized (see text) phase response of the potential oscillations in the elongating regions of several plants to changes in period of (a) osmotic pressure oscillations and (b) IAA oscillations.

are displaced somewhat with respect to one another although each still shows the same phase change from short to long period. The curves in Figure 5(b), however, are the most common type for the elongating or actively resonant region. Consequently to clarify the comparison between phase curves for a number of different plants, they have been normalized so that the phase angle at long periods is $+180^\circ$. This comparison of such normalized data is shown in Figure 6, different symbols being used to denote different plants. Figures 6(a) and 6(b) again show the contrast between the phase responses to oscillations in osmotic pressure and IAA concentration.

In all the experiments described so far a solution of 10^{-4}M KCl was used as the roots' bathing medium, the oscillations in sucrose or mannitol concentration (for osmotic pressure) and IAA concentration being superimposed on the constant background of 10^{-4}M KCl solution. In some cases the plant was allowed to equilibrate in this KCl bathing solution for 15 hr or more before the commencement of experiments. The phase and amplitude responses of these plants to oscillations in osmotic pressure and IAA concentration were the same as those for plants which were allowed

to equilibrate for only about 1 hr after removal from the tap water culture medium. This suggests that the bioelectric responses described are independent of the presence or absence of various ion species left in the root tissue, originally obtained from the tap water culture medium. However, it is possible that in both cases (short and long equilibration time in 10^{-4}M KCl) a variety of ion species from the cotyledon material was available to the root throughout the experiments.

In some series of experiments plants were first equilibrated in 10^{-4}M CaCl_2 for 15 hr or more before the oscillations in osmotic pressure or IAA concentration were applied, the background solution still being 10^{-4}M CaCl_2 . The amplitude and phase responses to osmotic pressure and IAA oscillations were the same under these conditions as those already described in which KCl was used throughout.

These results suggest that the oscillatory bioelectric currents and the physiological system responsible for the potential oscillations are not dependent on whether the cation in the bathing solution or the root tissue is monovalent or divalent. The effect of different anions has not been studied.

IV. DISCUSSION

The simplest system which exhibits properties similar to those of bean roots which have been described in this paper and by Scott (1957) is a feedback loop containing three linear, exponential-delay elements. As the authors are not aware of any simple treatment in the literature of this feedback system, and because it may have wider biological application, it has been thought desirable to consider the theory of it in some detail. This is done in Appendix I.

It will be seen that this feedback model has many of the properties which are exhibited by the plant root. It may be stable (i.e. any oscillations produced as a result of stimulation are transient and die out after a few cycles) or may oscillate spontaneously depending on the value of the feedback loop gain K (and also on the relative values of the three time delays). When a forcing oscillation is applied at any point in a stable loop, oscillations of the same period (although differing from it in phase) are caused at other points in the loop. The amplitude of the response to an applied oscillation depends on its period and resonates close to the natural period of the system. It is also found that changes in period of the applied oscillation cause the phase difference between it and the resulting oscillation at different points in the loop to change. The total change in phase in going from very low to very high periods is 180° if there are two delay elements between the point of application of the forcing oscillation and the point of observation; and 270° for three interposed delay elements.

Since all these properties are observed in the behaviour of the plant root, it is considered to be strong evidence that a feedback system of this kind is in operation in the plant.

It is not claimed that the actual processes in the plant can be described in detail in terms of the simple model considered above. There may be more elements and more complex modes of interaction. There is, for example, some evidence for a second oscillation with a natural period of about 90 min which would require an additional

feedback path. In addition the elements are unlikely to be truly linear or to have delay characteristics that are truly exponential. More complex delay elements would raise the order of the differential equation and would, in general, result in oscillations which were not sinusoidal. Since the oscillations produced by the plant are very nearly sinusoidal it is assumed that the elements have characteristics similar to those considered in the model.

A physiological feedback loop for the plant root is now proposed and is shown in Figure 7. This is essentially the same as that proposed by Scott (1957) but has been modified in some respects to fit the results described in this paper.

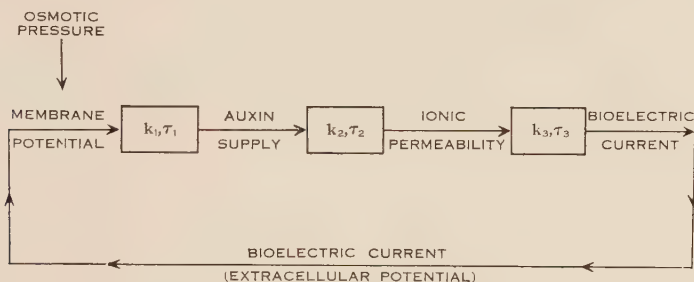


Fig. 7.—A proposed feedback loop with three exponential delays.

It is proposed that osmotic pressure is linked closely with the membrane potentials of the root's outer cells (i.e. epidermal and cortical cells) so that a change in osmotic pressure causes immediate changes in membrane potential. Experimental evidence for this assumption will be discussed in a further paper in this series when intracellular potential responses will be described.

It is proposed then that the electric fields across cell membranes are responsible for the distribution of auxin (IAA) within individual cells or groups of cells with a time-delay constant τ_1 . Transport of auxin by bioelectric fields has been proposed by a number of investigators (cf. Schrank 1957), but has been criticized on the grounds that these fields are not sufficiently large. Clarke (1937) has shown that there is no electrophoretic transport of IAA through agar blocks unless the applied fields are greater than 50 V/cm. This does not appear to be a valid criticism for, although the average bioelectric fields in bulk tissue are less than this value by several orders of magnitude, the fields in biological membranes are much larger. For instance, a potential of 1 mV across a membrane 100 Å thick results in an electric field of 10^5 V/cm in the membrane. Such fields are consequently quite sufficient to cause local electrophoretic movement of auxin (e.g. from one side of a membrane to the other), although other processes may well be involved in its movement through bulk tissue.

As the next stage is the feedback loop it is proposed that auxin modifies the properties of cellular membranes in their permeability to ions (time delay τ_2). In a considerable variety of tissues the effect of auxin on membranes permeability to water, ions, and other solutes has been studied. The sensitivity of membrane

permeability to auxin in such plant tissues as bean endocarp, the abscission zone of *Coleus*, and the leaves of *Mesembryanthemum* sp. and *Rhoeo discolor* has been reported by Sacher (1957, 1959) and by Sacher and Glasziou (1959). Ling and Gerard (1949) showed that IAA at $2.5 \times 10^{-4}M$ and $5 \times 10^{-3}M$ increases the permeability to potassium in the *Rana pipiens* sartorius fibre membrane. The effect is reversible on removal of the IAA. Bennet-Clark (1955) has suggested that the effect of auxin on the ionic permeability of plant cell membranes is exerted through an acetylcholine-mediated system.

The change in ionic permeability of plant cell membranes, affected by a change in auxin concentration or supply, may be caused primarily by auxin-induced cell wall plasticity. In decapitated *Avena* coleoptiles this cell wall plasticization occurs within a few minutes after the auxin addition (Adamson, personal communication). This in turn permits the uptake of water by the cell, stretching the plastic cell wall and the cell membrane enclosing the protoplasm (Adamson and Adamson 1958; Van Overbeek 1959). This mechanical stretching of the cell membrane could change its permeability to ions and other solutes.

This type of interaction between auxin and membrane permeability is in accordance with the observation that the actively resonant region of the root is situated in the elongating zone where the cell walls are most sensitive to auxin.

It is possible that variation in permeability could arise in the auxin-sensitive Donnan system of the cell wall (Van Overbeek 1959). This seems unlikely since the constitution of this system depends on whether it contains divalent or monovalent cations, whereas the bioelectric effects were found to be independent of whether the root's bathing solution contained monovalent (K^+) or divalent (Ca^{++}) cations. However, the concentration of these ions in the external solution may have been too low to alter appreciably the ionic composition of the cell wall.

The bioelectric currents which flow in developing biological tissue and through any surrounding conducting media are due to the non-uniform properties of this tissue. As auxin-induced permeability changes are likely to differ in different parts of the root it would be expected that these would modify ionic fluxes in the tissue and hence the magnitude and paths of bioelectric current. This is the final element in the proposed feedback loop and introduces a third delay, τ_3 .

To complete the feedback loop it is suggested that the bioelectric current would modify potential differences across membranes within the plant with negligible time delay. Evidence for this is available in experiments on the large algae (cf. Findlay 1959) and is to be expected as an ohmic property of any resistive membrane or barrier. Thus the loop is completed.

From Figure 7, it is apparent that between the auxin supply and the bioelectric current (or the extracellular bioelectric field) there are two delay elements, and three delay elements between the osmotic pressure and the bioelectric current. Thus the phase and amplitude relations for this physiological model are in agreement with those for the plant which have been described in this paper. It is, however, probable that other physiological feedback loops could be envisaged which would be equally in accord with the results described, but the one discussed above appears to be the simplest.

Applying this model to the results obtained and assuming that the three time constants are approximately equal gives values of the feedback loop gain K which range from about -2 to about -5 for the plants investigated, corresponding values of the time constants being in the range 0.8 – 1.4 min.

V. ACKNOWLEDGMENT

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APPENDIX I

ANALYSIS OF FEEDBACK LOOP CONTAINING THREE LINEAR,
EXPONENTIAL-DELAY ELEMENTS

This section presents a simplified analysis of the properties of a feedback loop containing three linear, exponential-delay elements. It will be shown that this system has properties similar to those exhibited by the plant root which have been described in this paper. The arrangement of elements is shown in Figure 8(a).

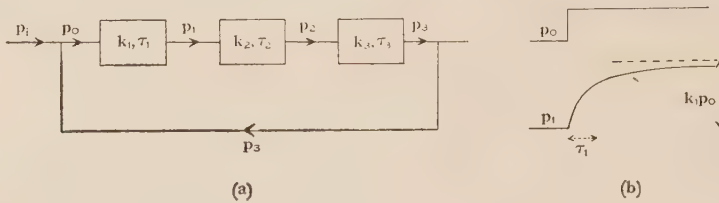


Fig. 8.—(a) The three-element feedback loop discussed in the text. (b) Illustrating the response p_1 to a step change in the input p_0 to the first delay element. p_1 changes to within $1/e$ of its final value ($k_1 p_0$) in a time equal to the time constant τ_1 .

A disturbance p_0 of the input quantity fed to the first delay element results in a disturbance p_1 in the output quantity. This is fed to the second delay element and then in turn to the third element. The output p_3 from this element is fed back so as to contribute to the input to the first element. The loop may have in addition an external input p_i .

The relation between the input and output of any element is characterized by constants k and τ . If the input to the first element suddenly changes by p_0 , the output p_1 changes exponentially to a final value $k_1 p_0$ with a time constant τ_1 (see Fig. 8(b)). That is,

$$p_1 = k_1 p_0 [1 - \exp(-t/\tau_1)],$$

or in differential form,

$$p_1 + \tau_1 (dp_1/dt) = k_1 p_0.$$

Such a relationship characterizes a linear, exponential-delay element. The constant k_1 may be either positive or negative depending on whether the output tends to increase or decrease when the input increases.

The equations describing the three-element loop (Fig. 8(a)) are as follows:

$$\left. \begin{aligned} p_1 + \tau_1 (dp_1/dt) &= k_1 p_0, \\ p_2 + \tau_2 (dp_2/dt) &= k_2 p_1, \\ p_3 + \tau_3 (dp_3/dt) &= k_3 p_2, \\ p_0 &= p_i + p_3. \end{aligned} \right\} \quad (1)$$

From these equations is obtained a differential equation of the third order for any of the quantities of the loop (such as p_3) in terms of p_i . Thus

$$\tau_1 \tau_2 \tau_3 (d^3 p_3 / dt^3) + (\tau_1 \tau_2 + \tau_2 \tau_3 + \tau_3 \tau_1) (d^2 p_3 / dt^2) + (\tau_1 + \tau_2 + \tau_3) (dp_3 / dt) + p_3 (1 - K) = K p_i, \quad (2)$$

where $K = k_1 k_2 k_3$ and is called the *feedback loop gain*. The feedback is positive or negative depending on whether K is positive or negative.

The solution of this equation is the sum of two terms: (i) a *complementary function* characteristic of the feedback loop alone (i.e. the solution of the equation when $p_i = 0$), and (ii) a *particular integral* which depends on the form of the input quantity p_i . The complementary function determines transient behaviour, while the particular integral gives the steady-state behaviour.

For simplicity the mathematical analysis is now restricted to the special case for which $\tau_1 = \tau_2 = \tau_3 (= \tau)$. No serious loss of generality results from this. The differential equation then becomes

$$\tau^3(d^3p_3/dt^3) + 3\tau^2(d^2p_3/dt^2) + 3\tau(dp_3/dt) + p_3(1-K) = Kp_i. \quad (3)$$

(i) *The Complementary Function* (i.e. the solution when $p_i = 0$)

The complementary function may be written in the form

$$p = P_a \exp(\alpha t/\tau) + P_b \exp(\beta t/\tau) \cos[(2\pi t/T) + \phi], \quad (4)$$

where p refers to any of the quantities p_1 , p_2 , and p_3 . P_a , P_b , and ϕ are arbitrary constants determined by the initial conditions, and α , β , and γ satisfy the relationships

$$\left. \begin{aligned} \alpha + 2\beta &= -3, \\ \gamma^2 + 2\alpha\beta &= 3, \\ \alpha\gamma^2 &= K - 1, \end{aligned} \right\} \quad (5)$$

and the natural period of the oscillation, T , is given by

$$T = 2\pi(\gamma^2 - \beta^2)^{-\frac{1}{2}}. \quad (6)$$

For a stable feedback loop (i.e. one in which any transient disturbance eventually dies out) it is evident from equation (4) that both α and β must be negative. This is found from equations (5) to be the case provided the loop gain K lies between $+1$ and -8 . For more positive values of K the first term increases exponentially, and for values of K less than -8 the oscillatory term increases in amplitude exponentially. In a practical control system the quantity fed back must be amplified (i.e. $K > \pm 1$); hence for stability the feedback must be negative and within the range of K values -1 to -8 . If the negative feedback gain is too large the system "hunts" (i.e. produces uncontrolled oscillations).

The degree of stability of the oscillatory component is measured by the decrement, Δ , which is the ratio of successive maximum disturbances. When $K = -8$, $\Delta = 1$ (i.e. oscillations are sustained), and Δ is zero (i.e. the system is critically damped) if $K = 1$. The values of Δ for $K = -2$ and -7 are 0.08 and 0.86 respectively.

If the time constants are not all equal the solution of the differential equation is of the same form, but the range of values of the loop gain K for stability is different. Typical values are set out below. The stable range is smallest when the time constants are all equal.

Relative Values of Time Constants	Range of Values of K for Stability
1 : 1 : 1	+1 to - 8.0
1 : 2 : 2	+1 to - 9.0
1 : 5 : 5	+1 to - 14.4
1 : 10 : 10	+1 to - 24.2
1 : 2 : 4	+1 to - 11.2
1 : 4 : 16	+1 to - 26.6
1 : 10 : 100	+1 to -122.2

Thus it is seen that a stable feedback system may become unstable either if the negative feedback gain increases, or if the relative values of the time constants change so that they are more nearly equal.

(ii) *The Particular Integral* (i.e. the steady-state behaviour)

The particular integral depends on the form of the input function p_i . For a sinusoidal input

$$p_i = P_i \exp(j\omega t),$$

and steady-state solutions of the form

$$p_3 = P_3 \exp[j(\omega t + \epsilon_3)],$$

are obtained for each of the quantities p_3 , p_2 , p_1 , and p_0 .

Substituting for p_3 and p_i in equation (3),

$$[\tau^3(j\omega)^3 + 3\tau^2(j\omega)^2 + 3\tau(j\omega) + (1-K)]P_3 \exp[j(\omega t + \epsilon_3)] = KP_i \exp(j\omega t),$$

from which is found by collecting real and imaginary parts that

$$P_3 = [KP_i/(A^2 + B^2)] \text{ and } \epsilon_3 = \text{artan}(-A/B),$$

where

$$A = 3\tau\omega - \tau^3\omega^3,$$

and

$$B = 1 - K - 3\tau^2\omega^2.$$

The amplitudes and phase relationships for p_2 , p_1 , and p_0 are now obtained easily from p_3 using equations (1). For sinusoidal oscillations of angular frequency ω ,

$$P_0/P_1 = P_1/P_2 = P_2/P_3 = (1 + \tau^2\omega^2)/k,$$

and there is a phase lag of $\text{artan}(\tau\omega)$ in each element. If the gain k for an element is negative, this can be expressed as an additional phase shift of π .

Typical graphs of the amplitude and phase relationships for the steady-state values of p_0 , p_1 , p_2 , and p_3 as functions of the period of the sinusoidal input p_i are shown in Figure 9. The cases considered are for loop gains of -2 and -7 . It is here assumed that the phase reversal necessary to make K negative occurs in the third element, but it is evident that it could occur in any one of the elements in the loop or in all three of them.

It is noted that resonance behaviour occurs in the vicinity of the natural period throughout the loop and this is more marked when K is -7 (i.e. when the system is approaching the unstable condition for which $K = -8$). The phase changes most rapidly with period in the vicinity of resonance, the effect again being more marked for $K = -7$ than for $K = -2$.

An important quantity for the present paper is the total change in phase (relative to the input) at various points in the loop when the input oscillation changes from very short period to very long period. The graphs show that this quantity (to be called the *total phase shift*) has values 0 , $\pi/2$, π , and $3\pi/2$ for p_0 , p_1 , p_2 , and p_3 respectively. The total phase shift therefore indicates the number of delay elements between the input oscillation and the point of observation of the forced oscillation, each element contributing $\pi/2$ to the total phase shift.

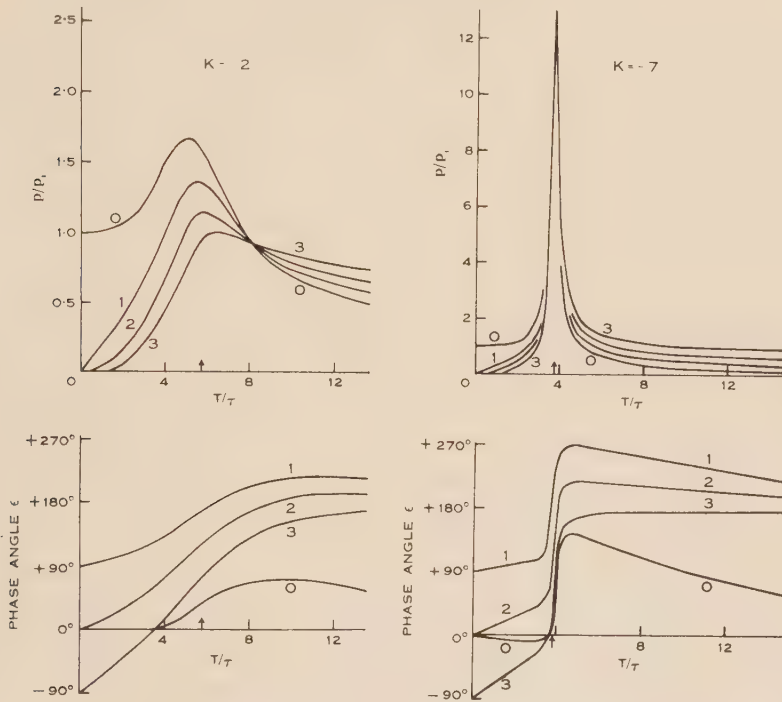


Fig. 9.—Amplitude and phase relationships for a sinusoidal input p_i at different points in the loop for two values of the loop gain, -2 and -7 . The numbers on the graphs refer to the various values of the amplitude $p(p_0, p_1, p_2, \text{ and } p_3)$, and phase angle $\epsilon(\epsilon_0, \epsilon_1, \epsilon_2, \text{ and } \epsilon_3)$.

All the features described above for the steady state apply equally well to loops in which all time constants are not equal. In applying the rule concerning the total phase shift, it must be remembered that the periods of the forcing oscillations must extend well beyond, both above and below, the range of time constants in the loop.

SERUM β -GLOBULIN POLYMORPHISM IN MICE

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Summary

Data are presented to show that there are three serum β -globulin types in laboratory mice controlled by a pair of alleles. Each allele appears to give rise to three electrophoretically distinct zones in starch gel. Within the inbred strain A/AGS there was variation between mice in the intensity of staining of the three zones. Reciprocal mating data gave no evidence of an effect of β -globulin type on segregation ratios as has been reported for cattle.

I. INTRODUCTION

Following the initial recognition of serum β -globulin polymorphism in cattle independently by Ashton (1957, 1958c), Hickman and Smithies (1957), and Smithies and Hickman (1958), it was soon established that the same phenomenon occurs in other mammals. Thus Smithies (1957) showed that human β -globulins are polymorphic while Ashton (1958d, 1958e, 1960a) and Ashton and McDougall (1958) demonstrated β -globulin polymorphisms in the serum of a number of farm animals including sheep, horses, goats, and pigs. Recently McDougall (personal communication) has found the phenomenon in red deer.

Other examples of serum protein polymorphism have been found. The original demonstration of qualitative inherited differences between the serum proteins of individuals was with the haemoglobin-binding α -globulins, termed haptoglobins (Smithies 1955). Polymorphic differences have also been found in the "thread-proteins" of cattle (Ashton 1958a) and pigs (Ashton 1960a), in the S α proteins of cattle, and in the α -globulins and pre-albumins of horses (Ashton 1958b, 1958d). However, while all mammals so far examined have shown β -globulin polymorphism, the other polymorphisms have been seen in only one or a few species.

Relationships between β -globulin type and fertility (Ashton 1960c) and β -globulin type and economic factors (Ashton 1960b) have been examined in dairy cattle. It was found that matings between homozygous cows and homozygous bulls were significantly more fertile than matings between heterozygous cows and heterozygous bulls, a finding confirmed by Ogden (personal communication). Also it was shown that β -globulin type affected milk yield, β^{DD} cows being on average superior to β^{AA} cows by about 50 gal. These observations prompted a search for similar effects in other species. For this reason the serum β -globulins of another ruminant, the sheep,

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are being investigated. It was also considered likely that the mechanisms underlying these β -globulin effects would be more easily identified in an animal more amenable to experimentation than the cow. Accordingly serum β -globulin polymorphism has been looked for, and found, in laboratory mice, guinea pigs, and rabbits. Laboratory mice have been studied in some detail, and the results are presented below.

The genetics of β -globulin polymorphism seems fairly simple and similar for each species. In each there appears to be a single locus controlling serum β -globulin type. So far, 5 alleles have been recognized in cattle (Ashton 1959b), 8 in the human (Giblett, Hickman, and Smithies 1959), 9 in sheep (Ashton and Ferguson, unpublished data), 2 in goats (Ashton and McDougall 1958), and 2 in pigs (Ashton 1960a). These results provided a baseline for the investigation of mouse β -globulin polymorphism.

II. MATERIALS AND METHODS

(a) *Strains of Mice Examined*

Sera were examined from mice of the inbred strains C57BL/AGS, CBA/AGS, A/AGS, and 101/AGS. The number of animals tested from each strain was 27, 26, 27, and 4 respectively. In addition mice from three outbred stocks were examined: a randomly bred albino stock from the Department of Veterinary Physiology, University of Sydney, the Laboratory Animals Bureau's grey stock, and +T, +t³, t³t³, and +t¹² mice derived from Tt³ and Tt¹² mutant lines maintained by Dr. S. Gluecksohn-Waelsch, New York. The number of mice examined from these three stocks were 26, 16, and 24 respectively. Three wild mice caught at Prospect were also tested.

Special mating groups were set up using mice of known serum β -globulin phenotype as indicated in Tables 1 and 2. Account was taken of the number of progeny born and the number weaned and, where possible, the phenotype of every mouse weaned was established. The mice were not tested until they had reached at least 5 weeks of age. Of those born 85% were weaned, and 78% were classified according to serum β -globulin type. Blood was obtained from the tail by the method described by Adams (1960), which enables samples of 0.5 ml to be taken rapidly; 20 mice could be bled in 30 min.

(b) *Starch-gel Electrophoresis*

Determination of the serum β -globulin phenotype was made by starch-gel electrophoresis using the horizontal technique developed by Smithies (1955) with minor modifications to the apparatus. The gels were prepared from hydrolysed starch (purchased from the Connaught Laboratories, University of Toronto, Canada) using phosphate buffer at pH 7.6 as described elsewhere (Ashton 1958c) for cattle serum proteins. Alternatively, we have used the discontinuous buffer system of Poulik (1957) as modified by Dr. K. A. Ferguson. With this system the electrolyte in the electrode compartments is a solution containing 1.2 g of lithium hydroxide and 11.8 g of boric acid per litre. The gel was prepared with a buffer made by adding 90 volumes of a solution containing 1.6 g citric acid and 6.2 g tris(hydroxymethyl)aminomethane per litre to 10 volumes of electrolyte. With this system and an applied voltage across the gel of about 10–12 V/cm the β -globulin zones were effectively separated in about 3 hr. The serum samples were inserted on rectangular pieces of filter paper

1 cm wide. To avoid irregularity in the final pattern the paper inserts were removed 15 min after the electrophoresis commenced. Several samples and a reference sample were run side-by-side on each gel. After electrophoresis the gels were slit lengthwise and the two exposed surfaces stained with nigrosine (Ashton 1958c). The β -globulin phenotype was assessed by comparison of the patterns given by the samples with that of the reference serum on the same gel.

The characterization of the zones separated from mouse serum by one-dimensional electrophoresis was aided by the two-dimensional electrophoresis technique of Smithies and Poulik (1956), first in agar in borate buffer (Ashton 1958c) and then in starch gel in phosphate buffer.

TABLE 1
SEGREGATION OF MOUSE SERUM β -GLOBULIN TYPES

Parental Phenotypes	No. of Offspring of Phenotype		
	β^{AA}	β^{AB}	β^{BB}
$\beta^{AA} \times \beta^{AA}$	*	—	—
$\beta^{AA} \times \beta^{AB}$	70	75	—
$\beta^{AA} \times \beta^{BB}$	—	91	—
$\beta^{BB} \times \beta^{BB}$	—	—	*
$\beta^{BB} \times \beta^{AB}$	—	167	172
$\beta^{AB} \times \beta^{AB}$	6	15	5

* Matings between homozygotes were not specifically examined, but numerous examinations from animals of several strains (inbred and outbred) revealed only one type within each strain.

III. RESULTS

(a) Serum β -Globulin Types

Plate 1, Figure 1, shows three β -globulin phenotypes which were found during this work. Phenotype β^{BB} occurred as the sole β -globulin type in the inbred strains C57BL, A, and 101, in three outbred stocks of different origin, and in the three wild mice tested, while phenotype β^{AA} was seen in only one strain (CBA), where it was the sole type. The third phenotype was produced by crossing animals of phenotypes β^{AA} and β^{BB} . The progeny were all of the third phenotype β^{AB} . These results, and the progeny totals from the various mating types shown in Table 1 make it clear that β -globulin polymorphism in the mice examined is controlled by two alleles which we have called β^A and β^B , so that genotypes β^A/β^A , β^A/β^B , and β^B/β^B are represented by phenotypes β^{AA} , β^{AB} , and β^{BB} . Further, each allele controls three protein zones as resolved by starch-gel electrophoresis: a fast-moving, rather faint zone not easily seen in all gels, an intermediate, more intensely staining zone, and a slow-moving, intensely staining zone. Although the point has not been checked the different degrees of staining probably represent corresponding variation of quantity of protein present in the three zones, rather than different binding capacities of the individual proteins for nigrosine.

Usually the relationship between the intensity of staining of constituent zones for any β -globulin allele within a given species remains reasonably constant. However, within the A/AGS inbred line we have seen two β^{BB} types (Plate 1, Figs. 2 and 3). Each gives three zones in starch gel, the mobilities of the three zones being identical for each

TABLE 2
SEGREGATION DATA FROM RECIPROCAL MATINGS

Parental Phenotypes		Mating Group No.	No. Litters	No. Born	No. Weaned	No. of Female Progeny		No. of Male Progeny	
						Homo-zygous	Hetero-zygous	Homo-zygous	Hetero-zygous
♀	♂								
β^{AA}	β^{AB}	3	2	15	14	3	4	1	6
		4	2	8	8	1	3	4	0
		5	4	18	17	4	1	1	4
		6	3	12	9	1	2	2	4
		21	2	17	13	5	3	3	2
		22	2	12	7	1	3	3	0
Total			15	82	68	15	16	14	16
β^{AA}	β^{AA}	1	5	30	30	7	6	6	9
		10	3	34	25	6	4	10	5
		11	4	29	23	5	6	3	5
		12	3	26	25	4	7	7	6
Total			15	119	103	22	23	26	25
β^{BB}	β^{AB}	7	11	100	88	23	23	25	14
		8	9	77	67	14	19	13	15
		9	10	99	85	22	21	24	17
Total			30	276	240	59	63	62	46
β^{AB}	β^{BB}	2	4	33	27	9	10	4	4
		13	3	29	27	10	4	7	5
		14	4	31	30	9	5	5	5
		15	4	43	34	4	10	3	15
Total			15	136	118	32	29	19	29
Totals			75	613	529	128	131	121	116

“subtype”. The most common type has zones which, in order of decreasing mobility, stain faintly, moderately, and intensely. The less common type has corresponding zones staining more or less evenly. When crossed with β^{AA} mice the resulting heterozygotes each showed three zones of corresponding mobility and were indistinguishable subjectively.

(b) Reciprocal Mating Data

Asymmetrical segregation ratios were obtained from matings between cattle of certain β -globulin genotypes (Ashton 1959a), so that there was a deficiency of offspring unlike the mother. This phenomenon was sought in mice.

Disturbed segregation ratios are most readily detected when two progeny phenotypes are expected in equal numbers from a mating type. Mice were therefore mated in four ways: $\beta^{AA}\text{♀} \times \beta^{AB}\text{♂}$, and its reciprocal $\beta^{AB}\text{♀} \times \beta^{AA}\text{♂}$; $\beta^{BB}\text{♀} \times \beta^{AB}\text{♂}$, and its reciprocal $\beta^{AB}\text{♀} \times \beta^{BB}\text{♂}$. The numbers of progeny observed of each phenotype is shown in Table 2. In none of the groups did the observed numbers differ significantly from the expected 1:1 ratio when tested by χ^2 , nor was there any evidence of a significant excess of offspring of one sex in the combined data.

IV. DISCUSSION

In common with other mammals the β -globulins of mice are polymorphic. As with all other examples of mammalian β -globulin polymorphism each allele appears to give rise to a group of zones on starch gel. In mice there seem to be three distinct zones per allele, in cattle four (Ashton 1959b), in sheep and goats two (Ashton and McDougall 1958), in horses three (Ashton 1958e), and in pigs three (Ashton 1960a). Recently Harris, Pennington, and Robson (1960) have demonstrated that there are probably two zones on starch gel for each β -globulin (transferrin) allele in the human.

It is possible that the β -globulin zones produced in each species from one allele are polymers of differing molecular size of a basic polypeptide. These would be separated in starch gel by the sieve-like action of the gel (Smithies 1955). However, the charge on each polymer would be the same, and supporting media not showing the sieve effect would not be expected to resolve the polymers. It has been demonstrated previously (Ashton and McDougall 1958) that while the β -globulin types in cattle, sheep, and goat sera may be recognized by paper electrophoresis, each allele gives rise on paper to one zone only, which cannot be resolved further.

The serum β -globulins of mice are unusual in that the relative staining intensities of the zones produced by an allele may differ between individuals within the same strain. Three individuals, showing the unusual pattern, from the A/AGS inbred line, were bled on two occasions about 2 weeks apart and the pattern had remained constant. The variation in relative staining intensity (and presumably in quantity of protein) of the zones in mice suggests a mechanism controlling the relationship between the constituent zones, although there is no evidence to distinguish between a genetic or physiological mechanism. The significance of the phenomenon is not known. However, it has been observed that in two sublines of the inbred strain, recognized by rejection of homografts, two individuals from one line had one β -globulin subtype, and two from the other line the other.

The data show no evidence of the disturbed segregation ratios that have been found in cattle, i.e. there is no consistent excess of female offspring of the same phenotype as the mother. It has been established that fertility in cattle is influenced by parental β -globulin type (Ashton 1960c), but that the mechanism is probably indepen-

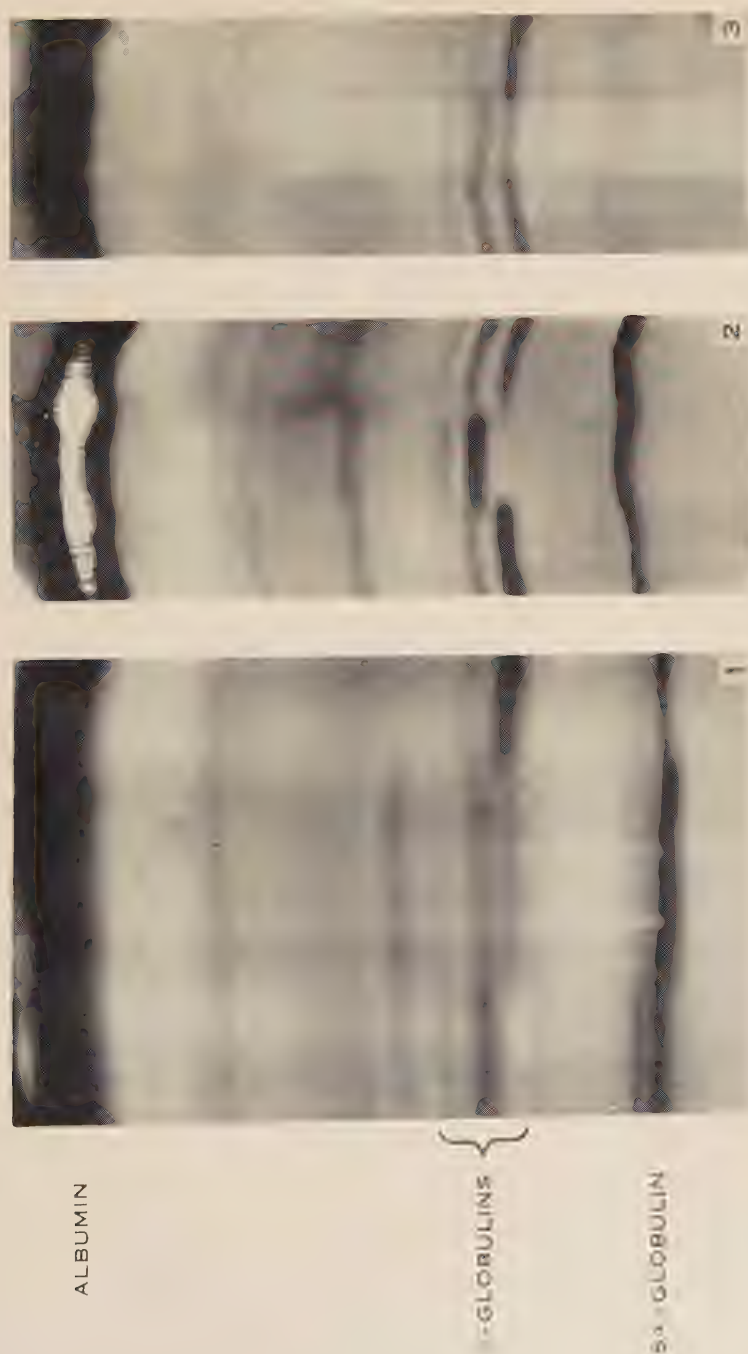
SERUM β -GLOBULIN POLYMORPHISM IN MICE

Fig. 1.—Anodic side of starch gel showing the stained protein zones from three different mouse sera after electrophoresis. The β -globulin phenotypes are, from left to right, β^{AA} , β^{AB} , and β^{BB} .

Fig. 2.—As for Plate 1, Figure 1, showing, from left to right, the phenotypes β^{BB} , β^{AA} , and modified β^{BB} . The latter phenotype illustrates clearly that each β -globulin allele produces three zones in starch gel.

Fig. 3.—As for Plate 1, Figures 1 and 2, showing, from left to right, the phenotypes β^{BB} modified, β^{AB} , and β^{BB} .

dent of that causing aberrant segregation ratios. The fact that the β -globulin mating groups in mice do not give asymmetrical segregation ratios does not necessarily mean therefore that parental β -globulin type has no effect on fertility.

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ON THE AMINO ACIDS ESSENTIAL FOR THE TISSUES OF THE SHEEP

By A. M. DOWNES*

[Manuscript received September 25, 1960]

Summary

A lactating ewe was injected intravenously with sodium [$1\text{-}^{14}\text{C}$]acetate and samples of the wool and milk produced subsequently were collected. Some of the casein from the milk produced 1-3 hr after the injection was hydrolysed and the distribution of ^{14}C among most of the amino acids determined. The results showed that only the following amino acids became labelled: glutamic acid, aspartic acid, proline, alanine, arginine, and serine. By analogy with earlier work on cows by Black *et al.* (1957) it was concluded that these amino acids are also non-essential for the tissues of the sheep.

The casein isolated from the milk produced during the first few hours had a much higher specific activity than the maximum observed in the wool. However, the results were consistent with the hypothesis that neither the wool keratin nor the casein were formed from the plasma proteins.

I. INTRODUCTION

Many living systems cannot synthesize certain of the amino acids required for growth or maintenance, at least in amounts adequate for the demands of anabolism. Rose (1938), in his studies of the nutritional requirements of certain mammals, called such amino acids which have to be supplied in the diet "essential". By the use of diets containing mixtures of highly purified amino acids in place of proteins he developed relatively simple and reliable methods for determining the nutritive significance of the individual amino acids. For example, by the "deletion" technique, in which single amino acids are successively removed from the complete diet, Rose, Oesterling, and Womack (1948) were able to show that for the growing rat 10 amino acids are essential dietary components—valine, leucine, isoleucine, threonine, methionine, phenylalanine, tryptophan, lysine, histidine, and arginine. However, it has been found that the results for the rat are not necessarily transferable to other species. Man, for example, requires the first eight only of the above amino acids for maintenance (Rose *et al.* 1955).

As an alternative to the deletion technique an indirect approach has been used. Steele (1952) showed that there was a striking absence of ^{14}C in the essential amino acids isolated from the mixed proteins of the carcass (with the entire gastrointestinal tract removed) of a mouse which had received a dose of [^{14}C]sucrose, even though the experimental conditions were favourable for the detection of possible minor metabolic routes to the essential amino acids through intermediates with low turnover rates. Recently Kasting and McGinnis (1958) used the indirect method, with [^{14}C]glucose, to determine the amino acids essential for an insect.

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This indirect approach has been particularly useful in the study of ruminants because nutritionally they do not require the essential amino acids to be supplied in their food. This is due to the presence of large numbers of microorganisms in the rumen which can synthesize enough of the essential amino acids to meet the needs of the animal (Loosli *et al.* 1949). There nevertheless remains the possibility that the tissue metabolism of ruminants differs from that of monogastric animals. To obtain a comparison between the two types of animals Kleiber and his co-workers have used the indirect method (Black, Kleiber, and Smith 1952; Black and Kleiber 1958). They gave intravenous injections of a number of ^{14}C -labelled compounds such as glucose and sodium bicarbonate, acetate, propionate, and butyrate to lactating cows and measured the amount of ^{14}C incorporated into the various amino acids of the casein. The amino acids examined could be divided into two groups—those with low levels of ^{14}C , which corresponded well with the essential amino acids, and those with much higher levels, which were in general the same as the non-essential amino acids. By injecting the compounds intravenously the rumen was largely by-passed. Thus the tissue metabolism of the cow was shown to be similar to that of the rat, dog and, except for histidine, man (Black, Kleiber, and Smith 1952).

There is apparently no published information on the amino acids essential for the sheep. However, some evidence has now been obtained by applying the indirect method outlined above. In this paper a comparison is made between some results obtained in the sheep and those published for the cow after an injection of sodium [$1\text{-}^{14}\text{C}$]acetate (Black *et al.* 1957).

II. EXPERIMENTAL

A lactating ewe (32 kg) was injected intravenously with sodium [$1\text{-}^{14}\text{C}$] acetate (50 mg; 555 μC). Just before the injection, and at intervals afterwards, the animal was milked and the wool clipped from a large area on each side. Just before the completion of each milking a dose of purified posterior pituitary extract (5 i.u. oxytocic; supplied by the Commonwealth Serum Laboratories, Melbourne), was injected and the animal milked dry.

(i) *Casein Samples*.—The casein was isolated from each sample of milk and purified as described by Askonas, Campbell, and Work (1954).

A portion (1 g) of the second casein sample was hydrolysed with HCl (200 ml, 6*N*) at 140°C for 20 hr. The phenylalanine and tyrosine were isolated from the hydrolysate as described by Partridge (1949) and the other amino acids eluted from a column (2.2 by 150 cm) of "Amberlite IR120" with increasing concentrations of HCl (Hirs, Moore, and Stein 1954).

Pure samples of all the amino acids were thus obtained except aspartic acid, serine, and the leucines. The aspartic acid and serine were separated on a column (0.8 by 40 cm) of "Deacidite FF" by elution with acetic acid after the method described by Hirs, Moore, and Stein (1954). No attempt was made to separate the leucines, since they contained practically no ^{14}C . The basic amino acids were kept as the hydrochlorides. The other amino acids were obtained as such by adding their

hydrochlorides to small columns of "Deacidite FF" and eluting with acetic acid (0.5N). The acetic acid solutions were evaporated to give the solid amino acids. The purity of each sample was checked by paper chromatography in *n*-butanol-acetic acid-water and in phenol-ammonia.

The casein and amino acid samples were either counted directly on 1-cm² polythene disks under an end-window G.M. counter, correcting the results to the infinite thickness value if necessary, or were combusted to CO₂ and counted as the gas as described by Bradley, Holloway, and McFarlane (1954).

(ii) *Wool Samples*.—The wool was washed with ether, ethanol, and water and dried in an oven at 110°C. Samples were counted directly as described by Downes (1961).

TABLE 1
SPECIFIC ACTIVITY OF SAMPLES OF CASEIN AND WOOL FROM A SHEEP AFTER AN
INTRAVENOUS DOSE OF SODIUM [1-¹⁴C]ACETATE

	Sample No.	Time of Collection Relative to Injection	Specific Activity (m μ c/g)
Casein	1	—52 min–68 min	527
	2	68 min–4 hr	219
	3	4–7 hr	83.8
	4	7–10.5 hr	36.6
	5	10.5–22.5 hr	15.9
	6	22.5–30 hr	9.1
Wool, left side	1	—1–4 days	1.6
	2	4–7 days	8.5
	3	7–11 days	12.4
	4	11–15 days	5.1
	5	15–19 days	2.1
Wool, right side	1	1–5 days	1.5
	2	5–8 days	15.0
	3	8–12 days	11.0
	4	12–15 days	6.7
	5	15–19 days	2.1

III. RESULTS

The specific activities of the samples of casein and wool are shown in Table 1. The first sample of casein had the highest specific activity, 0.527 μ c per g. Since this was obtained from the first milking, 68 min after the injection, the maximum specific activity must have been reached in the first few minutes. The later samples showed a rapid fall in specific activity. By comparison, much smaller amounts of ¹⁴C appeared in the wool, the maximum specific activity observed being 0.015 μ c per g in the wool grown from the fifth to the eighth day after the injection.

The specific activities of the individual amino acids from the second casein sample are shown in Table 2, together with the corresponding results obtained by

Black *et al.* (1957) for the amino acids from casein produced by cows during the first 3 hr after an injection of sodium [1-¹⁴C]acetate. For this comparison both sets of results are expressed as μC per g-atom of carbon divided by the number of micro-curies injected per kg of body weight.

TABLE 2
SPECIFIC ACTIVITY OF THE AMINO ACIDS ISOLATED FROM THE SECOND CASEIN
SAMPLE (SEE TABLE 1)

The amino acids whose counting rates were above background (8 counts/min) when counted at infinite thickness on 1-cm² polythene disks are compared in the last two columns with the corresponding mean results published by Black *et al.* (1957) for the casein from the milk of three cows

Amino Acid	Specific Activity (counts/min)	Specific Activity ($\mu\text{C/g-atom C}/\mu\text{C injected/kg body wt.}$)	
	Sheep	Sheep	Cow
Isoleucine*†	} 3		
Leucine*†			
Lysine*†			
Methionine*†			
Phenylalanine*†	1		
Threonine*†	7		
Tryptophan*†	—		
Valine*†	0		
Histidine†	0		
Arginine†	112	0.37	0.51
Glycine	75	0.30	0.42
Alanine	145	0.42	0.62
Serine	110	0.36	0.56
Cystine	—		
Tyrosine	2		
Aspartic acid	160	0.52	1.77
Glutamic acid	380	1.09	3.17
Proline	185	0.43	0.46

* Essential for maintenance of nitrogen equilibrium in normal adult man.

† Essential for optimum growth of young rats.

IV. DISCUSSION

The results obtained here for the sheep were substantially the same as those of Black *et al.* (1957) for the cow. In both cases the amino acids could be divided into the same two groups; firstly, tyrosine, phenylalanine, leucine, isoleucine, valine, methionine, threonine, lysine, and histidine, all of which had negligible radio-activity; and secondly, those containing ¹⁴C—glutamic acid, proline, aspartic acid, alanine, arginine, serine. Cystine and tryptophan were not isolated. Although there are individual variations, as would be expected in such a comparison, the specific activities were of the same order of magnitude as those published by Black *et al.*

(1957) for casein from cow's milk collected about 3 hr after a similar injection. These authors pointed out that only the non-essential amino acids became labelled significantly. Similarly, the present results suggest that at least 8 of the 10 amino acids essential for the rat are also essential for the tissues of the sheep. Arginine is evidently not essential for the sheep. No statement can be made about cystine or tryptophan since they were not isolated. Tyrosine is not classified as an essential amino acid from the nutritional experiments of Rose. However, since it is synthesized *in vivo* by the hydroxylation of phenylalanine, which is essential, no ^{14}C would be expected in the tyrosine unless the phenylalanine was radioactive also.

Casein is largely synthesized in rabbit and goat mammary glands from the free amino acids in the plasma rather than from large peptides or proteins (Barry 1958). If this also applies in the sheep, and the rapid fall in the specific activity of the casein in the present experiment suggests that it does, then a comparison of the specific activities of the amino acids in the casein and the wool from a sheep injected with labelled amino acids should throw some light on the mechanism of wool biosynthesis. Thus, if wool keratin is also derived from the plasma-free amino acids, the specific activity of any of its constituent amino acids should be the same as that of the same amino acid in the casein synthesized during the same time; if, on the other hand, wool keratin is formed from the plasma proteins, or from peptides derived from these proteins, the specific activity should be much lower in the wool than in the casein.

In the present experiment only a rough comparison of the wool and casein themselves, instead of individual amino acids, could be made. However, although these two proteins have different compositions, the total content of non-essential amino acids is about the same for each (Block and Bolling 1951), and it was calculated that the specific activity of wool derived from amino acids with the specific activities shown in Table 2 would be about the same as that of the casein.

From the specific activities of the casein samples given in Table 1 and the amounts of milk obtained (25 g/hr) it was calculated that the average specific activity of the casein produced during the first day after the injection was about 100 $\text{m}\mu\text{C/g}$. Since the specific activity had fallen to almost negligible proportions after the first day, the average value over the first 3 days must have been about 35 $\text{m}\mu\text{C/g}$, assuming a constant rate of milk production. The wool synthesized during the same period could not be clipped immediately because newly synthesized wool takes about 6 days to appear above the skin surface (Downes 1961). A maximum specific activity of 15 $\text{m}\mu\text{C/g}$ was observed in the 3 days' growth clipped 8 days after the injection. The true maximum attained in the wool must have been higher because, as pointed out previously (Downes 1961), the radioactive portions of the fibres cannot all be clipped simultaneously. The specific activity of the wool must therefore have approached that of the casein synthesized during the same time. This fact and the relatively rapid fall in the specific activity of the wool after the maximum was reached suggest that the plasma proteins are not intermediates in the synthesis of wool keratin. It must be emphasized that this tentative conclusion will have to be confirmed by detailed studies of the fate of labelled amino acids and plasma proteins in the sheep.

The results show that the tissue metabolism of the sheep is much the same as in other species. The accumulation of results of experiments similar to those reported here seems to be one of the few ways of comparing the nutritional needs of the tissues of the sheep with those of monogastric animals.

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STUDIES ON THE SODIUM-POTASSIUM BALANCE IN ERYTHROCYTES OF AUSTRALIAN MERINO SHEEP

II. OBSERVATIONS ON THREE MERINO STRAINS

By HELEN NEWTON TURNER* and JUDITH H. KOCH†

[Manuscript received September 27, 1960]

Summary

Estimations were made of the sodium-potassium balance in the erythrocytes of samples of ewes and rams and their progeny in three Australian Merino strains—two medium Peppin and one fine wool. In all three strains $[K^+]$ values occurred in a continuous distribution within a range of 6 to approximately 20 m-equiv/l packed red blood cells. In the fine wool strain there were no values beyond this, but in both medium strains animals were found with values in excess of 50. Means and variances of $[K^+]$ values, even excluding these extremes, were greater in the two medium strains. The variance was also significantly higher for females than males, though sex means did not differ significantly.

It is suggested that the presence of animals with extreme $[K^+]$ values traces back to the incorporation of some genes from a British breed into the medium strains. Further, the between-strain differences in variances when these extremes are excluded, combined with some between-sire differences, indicate that polygenes may be operating on the $[K^+]$ level as well as a pair of major genes which place it as high or low.

There is an indication that ewes with $[K^+]$ values near the mode have a better reproductive performance than ewes with values outside the modal class.

I. INTRODUCTION

As discussed by Koch and Turner (1961), these studies on the sodium-potassium balance in the erythrocytes of Australian Merino sheep were originally designed to investigate any possible association between potassium level in the erythrocytes and the ability of the lamb to survive, by comparing the $[K^+]$ values at birth for lambs which survived with those which did not. Previous work (Koch and Turner, loc. cit.) having shown that the $[K^+]$ levels at birth are all so high that classification into Evans' (1954) high and low categories is not feasible, this plan was abandoned. As an alternative, parents were sampled at mating to investigate the relationship between their $[K^+]$ values and the outcome of the mating.

As no information was then available on possible strain or sex differences in the distribution of $[K^+]$ values for individual sheep, sires and dams were sampled from three Merino strains at mating, male and female progeny resulting from these matings being sampled approximately 15 months later.

Estimates of both $[Na^+]$ and $[K^+]$ were made, but the present analyses are confined to $[K^+]$ values.

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II. MATERIAL AND METHODS

(a) *Experimental Sheep*

The sheep sampled came from two breeding experiments at the C.S.I.R.O. National Field Station, "Gilruth Plains", Cunnamulla, Qld. In one experiment, small closed families are under selection for high and low values of single characters, and in three pairs of these, under selection for body weight, staple length, and fibre population density, respectively, increased neonatal mortalities have been observed in the progeny of half-sib matings, indicating the possibility that some deleterious recessive gene might be present. The families are part of a large flock of medium Peppin Merinos, designated the G strain for this paper, whose general management has been described by Turner, Dolling, and Sheaffe (1959). Pen matings are used, and in these particular groups two sires are mated annually in each family, each sire to approximately 25 ewes of varying ages. During one mating period (May 1956), the ram and his ewes were sampled in one of the two groups in each family. No ram lambs in these families are castrated, and all offspring are retained, without selection, until at least 12 months of age. In August, 1957, when the progeny were 10-11 months old, samples were taken from all surviving offspring of the sampled parents.

In the second experiment, two of the Merino strains described by Roberts and Dunlop (1957) are used—the fine wool (F) and one of the medium Peppin strains (A), which is of different origin from the G strain. Parents in the second experiment are selected at random, and the majority of the ram lambs are castrated at marking. Otherwise management is similar to that of the first experiment. Six sires were mated in 1956 in the F and seven in the A strain, each to 15-20 ewes of varying ages. All sires and approximately 12 ewes from each sire group were sampled in the mating pens in May 1956. All surviving progeny of all sexes were sampled in August 1957, whether their dams had been sampled or not. As mating began a month earlier in the second experiment than in the first, the progeny were approximately a month older.

(b) *Environment*

The environment has been described by Turner, Dolling, and Sheaffe (*loc. cit.*). Cunnamulla lies on the plains of south-west Queensland, the district being one of high summer temperatures, with an average annual rainfall of approximately 15 in.

(c) *Determination of Sodium and Potassium Concentrations in Packed Red Blood Cells*

The techniques used for sampling and for the estimation of the sodium-potassium balance in the red blood cells were similar to those described by Koch and Turner (*loc. cit.*). The apparatus used in the present series was a Beckman model DU with a flame photometer attachment. All values of $[K^+]$ quoted are in m-equiv/l packed red blood cells.

(d) *Statistical Methods*

In some strains there were outlying values of $[K^+]$ which were so far beyond the nearest value that it seemed reasonable to regard them as belonging to a sub-population. Overall means including them were calculated, as well as means and

variances which excluded them. Even these variances differed between strains and sexes, and tests of the significance of differences between strain and sex means were carried out using logarithmic transformations, still excluding the outlying obser-

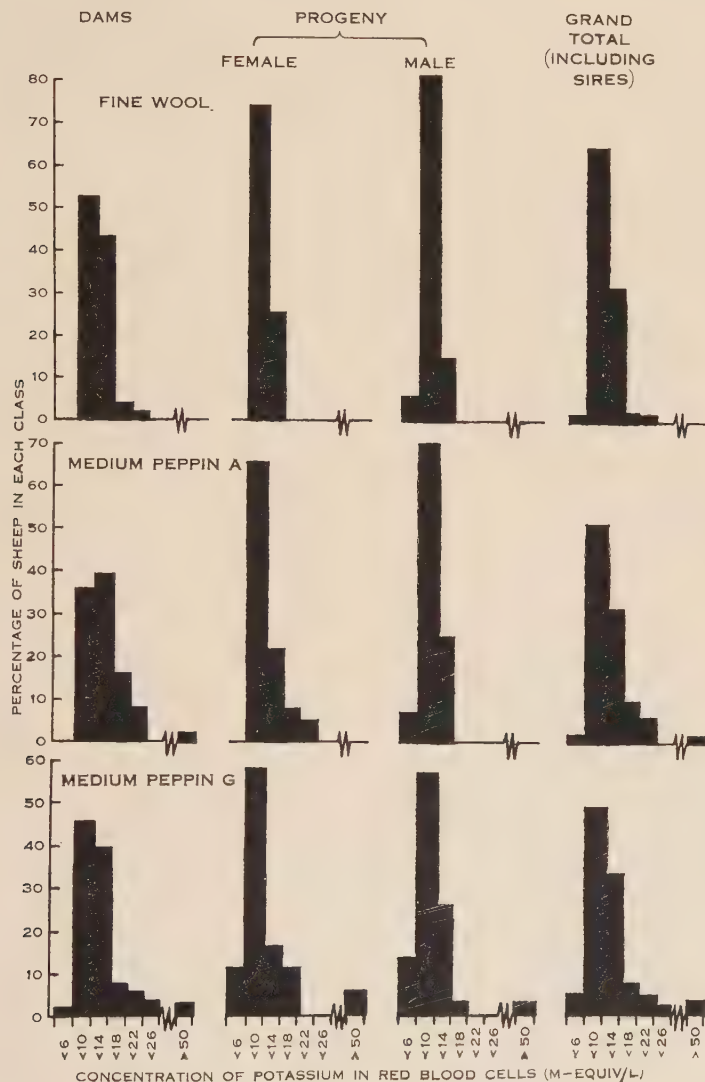


Fig. 1.—Frequency distribution of adult ewes and progeny of all sexes according to their $[K^+]$ values.

variations. Significance tests including these extreme values were carried out by χ^2 tests of the frequency distributions over class intervals of $[K^+]$ values. In most cases the groupings under 10, 10–14, and 14 or over were used.

Differences between ewes within a strain, classified according to their reproductive history in 1956, were analysed by testing weighted mean differences between subgroups within each sire group (Yates 1933). In this case original data were used, without transformation, as no unequal variances were involved.

TABLE 2
MEAN VALUES OF $[K^+]$ FOR PROGENY IN EACH SIRE GROUP
Original data

Strain	Sire	Number of Progeny		$[K^+]$ Values		
		Females	Males	Sire	Progeny Means*	
					Female	Male
Fine wool F	F1	5	2	6.8	9.8	7.0
	2	6	5	9.4	8.5	7.2
	3	4	4	7.1	10.3	9.6
	4	6	5	9.0	7.7	8.4
	5	5	5	7.3	8.0	7.1
	6	1	—	8.7	8.0	—
Medium Peppin A	A1	4	8	10.7	8.9	9.2
	2	9	5	14.1	11.2	9.5
	3	8	7	12.3	9.6	7.4
	4	2	—	20.6	7.7	—
	5†	4	9	11.2	7.4	8.6
	6†	10	1	70.0	12.9	12.6
	7	4	3	6.9	7.7	7.2
Medium Peppin G†	HB†	5	5	11.6	8.9	8.8
	LB	5	5	8.1	8.9	7.9
	HN	9	4	18.4	9.0	7.6
	LN	8	7	10.3	9.7	10.7
	HL†	5	8	13.3	10.1	8.9
	LL	4	9	19.4	6.8	8.8

* Excluding outlying values.

† Families selected for high and low body weight (HB and LB), fibre population density (HN and LN), and staple length (HL and LL).

‡ Outlying values: A6, the sire; HB, one female progeny; HL, one female and one male progeny. Four dams (not shown above) also had outlying values (1 in A5, 1 in HN, 2 in HL).

III. RESULTS

(a) Differences between Sexes, Sire Groups, and Strains

Frequency distributions of sheep are shown in Table 1, grouped in intervals of 4 m-equiv/l of potassium in the packed red blood cells. This interval was chosen because it appeared to give a smooth distribution. The four types of animals sampled

(adults and progeny of two sexes) are shown separately for each strain, together with the strain total. Only animals with complete data for $[\text{Na}^+]$ and $[\text{K}^+]$ have been included, a few with missing $[\text{Na}^+]$ values having been omitted. The frequencies, expressed as a percentage of the total number of sheep of the strain and sex under consideration, have been plotted in Figure 1.

Values of $[\text{K}^+]$ up to approximately 20 occurred in most subgroups in a continuous distribution, while in some subgroups of the two medium strains there were outlying values of over 50. Means including and excluding these outlying values are shown in Table 1, together with the standard deviations excluding them.

TABLE 3
VARIANCES OF $[\text{K}^+]$ WITHIN SIRE GROUPS FOR PROGENY OF EACH SEX IN EACH STRAIN*
Original data

Strain	Females		Males	
	Degrees of Freedom	Variance	Degrees of Freedom	Variance
Fine wool F	21	3.26	16	3.02
Medium Peppin A	34	9.76	27	3.39
Medium Peppin G	30	9.73	32	5.59

* Excluding three values over 50 m-equiv/l in the G strain.

The male progeny in the G strain were all rams, while all but five in the F and all but eight in the A strain were wethers. No differences between wethers and rams were apparent in these strains, and all male progeny were considered together.

The number of adult rams sampled was too small for an examination of sex differences, but this can be made in the progeny. Table 1 indicates that within each strain the female progeny show more of a "tail" than the male, the difference being expressed in a higher mean or variance, or both. As the sex ratio varied between sire groups, the comparison of sexes should be made within sire groups, and the mean value of $[\text{K}^+]$ for each sex in each sire group is shown in Table 2, together with the sire's own value. The within-sire variances are shown in Table 3.

On Bartlett's test (1937) the six variances in Table 3 differ significantly (corrected $\chi^2_5 = 18.64$, $P < 0.01$). The sex variances differ significantly (corrected $\chi^2_1 = 8.29$, $P < 0.01$) as do those for strains (corrected $\chi^2_2 = 8.76$, $P < 0.02$).

Table 4 shows the between- and within-sire analyses on the logarithms of the $[\text{K}^+]$ values. The within-sire variances no longer differ significantly ($\chi^2_5 = 9.03$, $0.2 > P > 0.1$), the pooled value over all sexes and strains being 0.0121.

Weighted mean differences between the sexes within sire groups, on logarithms, are 0.044 ± 0.026 , 0.032 ± 0.026 , and -0.004 ± 0.031 for the F, A, and G strains, respectively, the pooled value being 0.021 ± 0.016 .

The sex difference in variance indicated in Table 1 can therefore be regarded as significant, but the difference in mean values is not.

Strain differences can be examined both in adult ewes and in both sexes of the progeny. As indicated in Table 3, there were significant between-strain differences in variance of the original data in the progeny; as these were also present in the ewes (see Table 1), analyses of variance were carried out on logarithms (Table 5).

TABLE 4

ANALYSES OF VARIANCE OF LOGARITHMS OF $[K^+]$ VALUES† BETWEEN AND WITHIN SIRE GROUPS

Strain	Source of Variance	Adult Ewes		Progeny			
		Degrees of Freedom	Variance	Females		Males	
				Degrees of Freedom	Variance	Degrees of Freedom	Variance
Fine wool F	Between sire groups	5	0.0197	4	0.0148	4	0.0131
	Within sire groups	59	0.0094	21	0.0073	16	0.0090
Medium Peppin A	Between sire groups	6	0.0296	6	0.0433**	5	0.0173
	Within sire groups	55	0.0140	34	0.0118	26	0.0079
Medium Peppin G	Between sire groups	5	0.0635**	5	0.0148	5	0.0141
	Within sire groups	129	0.0150	30	0.0192	32	0.0140

† Excluding those values over 50 m-equiv/l.

** $P < 0.01$.

Table 5 gives pooled overall variances within strains, as well as a separation of these into between-sire and within-sire terms. Table 4 had indicated significant between-sire variances only for the ewes of strain G and the ewe progeny of strain A. For strains F and A the ewes were allotted to sires at random, and sire group differences among the dams would not be expected. In strain G, however, each sire group belongs to a separate closed family, and the ewes within one sire group are genetically related. Almost all progeny within one sire group are half-sibs, a very small number of full-sibs being included with them.

The pooled between-sire term over the three strains was significant for the adult and progeny ewes, but not for the males. It seemed reasonable to test the between-strains term against the overall variance rather than the between-sire

term, however, regarding all animals as the strain sample. This was done in each set of data (Table 5). The strain differences are then significant at the 1% level in the adult ewes, but not significant for the progeny, though for the ewe progeny the variance ratio was approaching significance ($0.1 > P > 0.05$).

Strain differences can also be assessed by means of χ^2 tests of the frequency distributions of Table 1, and in this analysis the outlying observations can be included. For the dams, there are significant strain differences ($\chi^2_4 = 11.06$, $P = 0.02$) arising chiefly from a marked difference between the F and the two medium strains. Each of the latter shows a "tail" to the right (Fig. 1) and contains

TABLE 5
ANALYSES OF VARIANCE OF LOGARITHMS OF $[K^+]$ VALUES† BETWEEN STRAINS

Source of Variance	Adult Ewes		Progeny			
	Degrees of Freedom	Variance	Female		Male	
			Degrees of Freedom	Variance	Degrees of Freedom	Variance
Between strains	2	0.0825**	2	0.0381	2	0.0155
Within strains	259	0.0149	100	0.0152	88	0.0114
Between sire groups within strains	16	0.0371**	15	0.0262*	14	0.0149
Within sires	243	0.0134	85	0.0133	74	0.0108

* $P < 0.05$.

** $P < 0.01$.

† Excluding values over 50 m-equiv/l.

a few animals with $[K^+]$ values in excess of 50. The F strain has only three ewes with values over 14, and none over 20. In all three strains, at least 75% of the ewes have $[K^+]$ values between 6 and 14.

For the progeny, the same trends in strain differences are present in both sexes, but in neither sex did the distributions differ significantly. [$\chi^2_4 = 5.09$ for females, χ^2_2 (grouping into under and over 10) = 2.04 for males, $P = 0.3$ approximately in each case.]

(b) Differences between Adult and Progeny Groups

In all three strains in Table 1, the ewe progeny show a lower mean for $[K^+]$ than the corresponding adult ewes. The weighted mean difference, on logarithms, is 0.073 ± 0.014 , giving a t_{358} value of 5.21 ($P < 0.001$).

The adult and progeny ewes of Table 1 are not all paired, since some adults failed to raise a lamb at all while others had male lambs, and additional progeny were sampled in some strains. To eliminate this source of variation, the daughter's value was compared with the mid-parent value in all dam-daughter pairs, which totalled 68 over the three strains. In 52 of these the daughter's value was lower than the mid-parent value, which confirms the discrepancy noted in Table 1.

This lowering of the average value in the progeny has not been explained. The progeny were approximately 10 months old, while the adults ranged in age from 2 to 10 years. Observations in the Merino by Koch and Turner (1961) indicated that $[K^+]$ values fell with age after birth, and appeared to reach a limit early in life, while Evans (1957) has always considered that a stable adult value for the sodium-potassium balance is reached before 6 months of age. Age differences thus do not seem to be a likely explanation of the fall in the progeny values.

The estimates on the two groups of animals were made at different times a year apart, and one possible explanation is some undetected difference in the technique on the two occasions. Such a difference, even if present, would not invalidate comparisons made within any one set of data.

(c) *Association between $[K^+]$ Values and Reproductive Performance of Ewes*

Evans and Mounib (1957) suggested that the sodium-potassium balance might have some bearing on survival in different environments. The number of ewes in the present series which fell into Evans' *HK* category (the over 50 class in Table 1) was too small to be informative; of the four ewes concerned, one raised a lamb to weaning in 1956 while the other three bore one lamb each but failed to rear it.

The strain differences in variance of $[K^+]$, even when the outlying observations are omitted, and the sire group differences in some strains, indicate additional genetic variation, however, and it seemed worth while to examine the possibility that differences in $[K^+]$ level, even within the range of the lower population, might have some bearing on survival. Since the lambs themselves could not be examined, the alternative was to compare the reproductive performance of ewes with different $[K^+]$ values.

Semen testing of rams is carried out each year just prior to mating, and in 1956 the semen pictures were much worse than usual; those for the G strain, which was mated a month later than the F and A strains, were slightly better but were still poor. From among the chosen sires and reserves those with the best semen pictures were finally mated, but even these were below the standard accepted in previous years, and the poor pictures were confirmed by a lowered lambing percentage. One factor contributing to the atypical ram performance might have been the weather conditions, the preceding summer having been a period of abnormally heavy rains and floods.

As a first step, however, the results of the 1956 matings were examined, ewes being classified on their 1956 performance as having raised (R) or failed to raise (N) at least one lamb to weaning. The N group included ewes which were dry, with or without a record of service at mating, as well as those which bore one or more lambs

but raised none. Ewes which were dry with a recorded service could have failed because of poor semen or because of intra-uterine loss, while ewes which were dry with no service recorded could have failed to come on heat through poor adaptation. To eliminate the influence of the sire, the means of $[K^+]$ values for R and N ewes within each sire group were compared, but the weighted mean difference over all strains was negligible.

TABLE 6
REPRODUCTIVE PERFORMANCE AND $[K^+]$ VALUES OF EWES PRESENT FOR FOUR OR THREE MATINGS

Strain	Number of Matings	$[K^+]$ Class* (m-equiv/l)	Number of Ewes which Raised:		Total No. of Ewes
			2 Lambs or Less	3 Lambs or More	
F	4	6 < 8	4	2	6
		8 < 12	11	15	26
		12 +	15	2	17
Total			30	19	49
A	4	6 < 8	3	2	5
		8 < 12	11	16	27
		12 +	11	11	22
Total			25	29	54
G	3	6 < 8	11	4	15
		8 < 12	20	19	39
		12 +	13	6	19
Total			44	29	73

* Excluding four ewes with values over 50 m-equiv/l. Their performance in lambs raised for a given number of matings was: strain A, 1/4; strain G, 0/3, 1/2, 0/1.

The results of further matings are now available for some of the sampled ewes, the F and A strains having been mated in three and the G strains in two later years.* Fresh sires were used in each strain each year, and the semen pictures since 1956 have improved considerably. The ewes in each strain were therefore classified according to the number of lambs raised in the total years of mating (4 or 3), disregarding sire groups.

Although there were differences between strains in mean $[K^+]$ values, there was a high concentration of values within a narrow range, and this gave rise to the idea that animals with a value within this range might have an advantage in fitness over those lying outside the range. To isolate these "tails" more precisely, ewes with records for the full 4 or 3 years were regrouped into $[K^+]$ classes of 6 and under 8,

* Drought precluded matings in the G strain in 1958.

8 and under 12, and 12 or over. Two-way tables for these $[K^+]$ values and reproductive performance are given in Table 6, the two "tails" being summed in Table 7 for comparison with the modal class.

Table 7 indicates that in all three strains more animals in the modal class than in the tails raised three lambs or more. The three 2 by 2 tables yielded χ^2_1 values of 6.74 ($P < 0.01$), 0.30 ($P = 0.60$), and 2.07 ($P = 0.15$) for the F, A, and G strains respectively, the summed value of χ^2_3 being 9.11 ($P < 0.05$).

The mean numbers of lambs raised per ewe per year are also shown in Table 7. The value for the modal class is higher in the F and A strains, but not in the G.

TABLE 7
REPRODUCTIVE PERFORMANCE FOR EWES IN THE MODAL CLASS* AND THE TAILS† OF TABLE 6

Strain	$[K^+]$ Class	Number of Ewes which Raised:		Number of Lambs Raised per Ewe per Year
		2 Lambs or Less	3 Lambs or More	
F	Modal class	11	15	0.64
	Sum of tails	19	4	0.38
A	Modal class	11	16	0.71
	Sum of tails	14	13	0.65
G	Modal class	20	19	0.74
	Sum of tails	24	10	0.77

* $8 < 12$ m-equiv/l.

† $6 < 8$ and $12 +$ m-equiv/l.

IV. DISCUSSION

The finding of strain differences in the distribution of $[K^+]$ values is of considerable interest. Evans and King (1955) have previously reported the existence of alleles differentiating the sheep population into classes with high and low values of $[K^+]$, the gene for *HK* being recessive to that for *LK*, while Evans and Mounib (loc. cit.) have shown that the gene frequency for *HK* varies from breed to breed. The absence of sheep with *HK* values from the fine wool strain agrees with the earlier conclusions of Bernstein (1954) and Evans (1957) that the Merino is characteristically an *LK* breed. In the two medium strains there is a clear separation into classes corresponding to *LK* and *HK*, the distribution of *HK* animals over sire groups being consistent with the hypothesis that *HK* is recessive to *LK*. The presence of such animals in these strains could be explained by an earlier introduction of animals from British breeds carrying a proportion of *HK* genes. It is of interest that of the six *HK* animals occurring in families of the G strain, four were in the family selected for long staple, which could well be a characteristic contributed by ancestors from a long-wool British breed. Further analyses of the association between the $[K^+]$ level and various fibre characteristics are planned.

In addition to having *HK* animals which were absent from the *F* strain, each of the two medium strains had a higher mean $[K^+]$ value for the *LK* animals than that of the *F* strain. Evans and Mounib (*loc. cit.*) found differences among the British breeds in the mean $[K^+]$ value for both *LK* and *HK* groups.

Another point of agreement with Evans and Mounib is the between-sheep variation in $[K^+]$ values within each *LK* and *HK* group. For the *LK* groups, this variation was greater in the medium strains than in the fine wool. The standard errors of *LK* values quoted by Evans and Mounib for British breeds, however, are comparable with our fine wool estimates. There was no strong evidence in our data for the bimodal distribution of the *Kea* group discussed by Evans (1957); as shown by Table 1 and Figure 1, the histograms are smooth on the classes shown, and very slight variations which appeared with finer groupings were regarded as arising from sampling variation.

The numbers of *HK* animals in our data are too small for a comparison of within-strain variances, but their variability, as indicated by the values listed at the foot of Table 1, appears in general to be greater than that of the *LK* group. This is also the case in the Evans and Mounib data.

One explanation of the variability in the *LK* group could be an incomplete dominance of the *LK* major gene, heterozygotes having a higher $[K^+]$ value than homozygotes, as suggested by Evans *et al.* (1956), who recorded a difference between known heterozygotes and a group including both hetero- and homozygotes. Approximately 2% of the adult ewes in the two medium strains were *HK*, giving a gene frequency under random mating of 14%, and an expectation of 24% of heterozygous animals. Such heterozygotes would be absent from the *F* strain, and their presence in the *A* and *G* strains could explain the higher variance which was observed; they would also explain Evans' (1957) bimodal distribution. There were no *HK* animals among the progeny of the only *HK* sire (A6, Table 2), so all must be heterozygotes, and their mean $[K^+]$ value is above that of all other sire groups.

However, variability among the *HK* values can certainly not be explained by the presence of heterozygotes, and in the breed data of Evans and Mounib the mean $[K^+]$ value of the *LK* group shows no association at all with the expected proportion of heterozygotes. As no haemoglobin analyses were made in our observations, the influence of haemoglobin type on the $[K^+]$ level could not be checked (Evans, Harris, and Warren 1958). Environmental influences may, of course, be producing variability, but the observed differences between means of breeds, strains, and sire groups indicate that at least some of the variation is of genetic origin, and it seems likely that polygenes are operating on the $[K^+]$ level as well as the major gene pair, as was suggested by Evans and Phillipson (1957) for cattle. The numbers of observations here are too small for reliable estimates of heritability, but it is hoped that some may be possible later.

There is some indication of an association between the $[K^+]$ value of a ewe and her reproductive performance. The number of *HK* ewes (four) was too small for any firm conclusions concerning this genotype, but their performance was poor, as indicated by the records of number of lambs raised from a given number of matings (Table 6), the average per ewe per mating being 0.20.

Among the *LK* ewes there is evidence of an optimum level of $[K^+]$; over the three strains, 54% of ewes with values from 8 to 12 m-equiv/l raised three lambs or more from three or four matings, compared with only 32% of ewes with values outside this range. The mean number of lambs raised per ewe per year was 0.70 for those in the modal class and 0.62 for those outside it. The differences are not large, but in view of the poor lambing performance of the Australian Merino any likely source of improvement is worth further exploration. Lambing records for the F, A, and G strains at Cunnamulla and for the F and A strains at other centres are available for a number of years, and analyses at present being made can be interpreted in the light of the observed differences in $[K^+]$ values between strains.

If, as appears possible, there is an optimum $[K^+]$ value for the Merino in Australia near the low end of the scale, one would expect, according to Waddington's theory of canalization, that selection would eventually reduce the variability about this mean value. This process may already have started, as there is an indication in Figure 1 that values are concentrated in a narrow range, but this is another point which requires more extensive data before anything definite can be concluded.

V. ACKNOWLEDGMENTS

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DIFFERENCES IN THE CONCENTRATION OF POTASSIUM AND THE TYPE OF HAEMOGLOBIN BETWEEN STRAINS AND SEXES OF MERINO SHEEP

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Summary

The concentration of potassium in, and the haemoglobin type of, the erythrocytes of five strains of Merino sheep have been determined.

The gene frequency for high potassium and haemoglobin type *A* in each strain has been determined. Strong non-Peppin Merino sheep had the highest gene frequency for high potassium (0.16) and the highest mean erythrocyte potassium concentration (approx. 15.7 m-equiv/l) as compared with 0.05 and approximately 12.0, respectively, for the other strains examined. A higher concentration of potassium in the whole blood of rams as compared with ewes ($P < 0.1$) was found.

The gene frequency for sheep haemoglobin type *A* was determined on four strains and a significantly lower frequency found in Peppin strains compared with non-Peppin strains.

The findings are discussed and their possible significance in relation to lambing percentages in the Merino outlined. The possibility that an erythrocyte potassium concentration of approximately 12.0 m-equiv/l, associated with type *A* haemoglobin is an advantage in sheep in Australia is suggested.

I. INTRODUCTION

The concentration of potassium in the whole blood of sheep, $[K_b^+]$, varies about two means, and these two sheep types in British breeds have been designated *HK* ($[K_b^+]$ approx. 35 m-equiv/l) and *LK* ($[K_b^+]$ approx. 12 m-equiv/l) respectively (Evans 1954). The two types occur in a wide variety of breeds of sheep (Evans 1956; Evans and Mounib 1957; Evans, Harris, and Warren 1958*a*, 1958*b*) and are due entirely to differences in the erythrocytes. The frequency of the gene responsible for animals with *HK* erythrocytes varies from breed to breed and is higher in mountain and northern European breeds than in lowland and African breeds (Evans and Mounib 1957; Evans, Harris, and Warren 1958*b*). Two red blood cell potassium types have also been found in goats (Evans and Phillipson 1957), possums (Barker 1958), and in the ox (Evans, unpublished data 1960). The mode of inheritance has been described (Evans and King 1955) and studies of the two types of sheep in relation to water metabolism (Evans 1957*b*), a number of husbandry criteria (King *et al.* 1958), other blood factors (Mounib and Evans 1959), and the concentration of potassium in the tissues (Mounib and Evans 1960) have been reported. The nature of the difference in the size of the potassium compartment has been studied (Joyce and Weatherall 1958; Tosteson and Hoffman 1958).

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Merino sheep have a low gene frequency for *HK* (Evans 1957*a*; Evans, Harris, and Warren 1958*b*) and *LK* Merino sheep differ from the great majority of *LK* sheep in British breeds in having much lower concentrations of potassium in their erythrocytes ($[K_e^+]$). The *HK* Merino sheep studied by Evans (1957*a*) appear to be more closely related to the *HK* types found in breeds of sheep indigenous to hot dry environments rather than to *HK* types in British breeds. The *HK* type may be divided into three subtypes which have been called *Keβ*, *Keγ*, and *Keδ*. *Keγ* is the normal *HK* subtype in British breeds. The overall geographic distribution of the *HK* gene (Evans 1956) has suggested an adaptive significance for the pair of alleles controlling $[K_e^+]$, and the difference in water metabolism between *LK* and *HK* sheep (Evans 1957*b*) has suggested that *LK* animals may have more chance of survival in relatively arid regions than *HK* animals. Bernstein (personal communication) has noted considerable variability in $[K_e^+]$ of South African Merino sheep and his mean value for *LK* animals in this breed (Bernstein 1954) is much higher than that found in Australian Merino sheep (Denton *et al.* 1951; Harris, McDonald, and Williams 1952; Evans 1957*a*). The true nature of the variability in $[K_e^+]$ of *LK* South African Merino sheep is therefore of considerable interest particularly when it is compared with the stability of $[K_e^+]$ of Scottish Blackface sheep kept under controlled conditions. It is important to know whether Bernstein's high mean value and variability are due to a marked variation about a normally high value, or due to circumstances peculiar to the time of examination when those sheep had values considerably in excess of the normal value of 12 m-equiv/l. Recent work (Evans, unpublished data 1960) suggests that the latter explanation may be the correct one, as fourfold increases in $[K_e^+]$ of *LK* animals not kept under standard conditions have been recorded.

The data reported here were collected so that the mean $[K_e^+]$ of *LK* Australian Merino sheep and the gene frequency for *HK* in this breed could be determined with some accuracy. It was intended to be a background upon which other experiments relating to the adaptive significance of erythrocyte electrolyte concentrations could be designed or interpreted. As similar breeds had shown in previous work different electrolyte patterns, and as strong non-Peppin and fine non-Peppin animals are distinct types and occupy recognizable ecological niches, differences between strains of Merino were theoretically possible and preliminary results from a group of experiments being carried out by Miss Helen Newton Turner and Dr Judith H. Koch, C.S.I.R.O., when the present experiments were started, and which were discussed with the author, suggested that differences did exist between strains and that the interpretation of the results of the experiments concerned with electrolyte concentrations in the erythrocytes of Merino sheep should take cognizance of this possibility.

The experiments were also undertaken so that *HK* Merino sheep could be found and used to form the nucleus of a flock of *HK* animals needed for the foundation of a flock of known homozygous *LK*, heterozygous *LK*, and homozygous *HK* Merino sheep.

A close association between sheep haemoglobin types and sheep red blood cell potassium types has been demonstrated (Evans *et al.* 1956), and as previous data (Evans, Harris and, Warren 1958*b*) had suggested that the Merino did not follow the

general pattern of British breeds in this respect, it was decided to investigate the haemoglobin types within the strains of Merino used in this investigation.

II. MATERIAL AND METHODS

(a) *Sheep*

Five strains of Australian Merino sheep, as described by Roberts and Dunlop (1957), were examined along with a large flock of fine-wool Merino sheep (Havilah) of

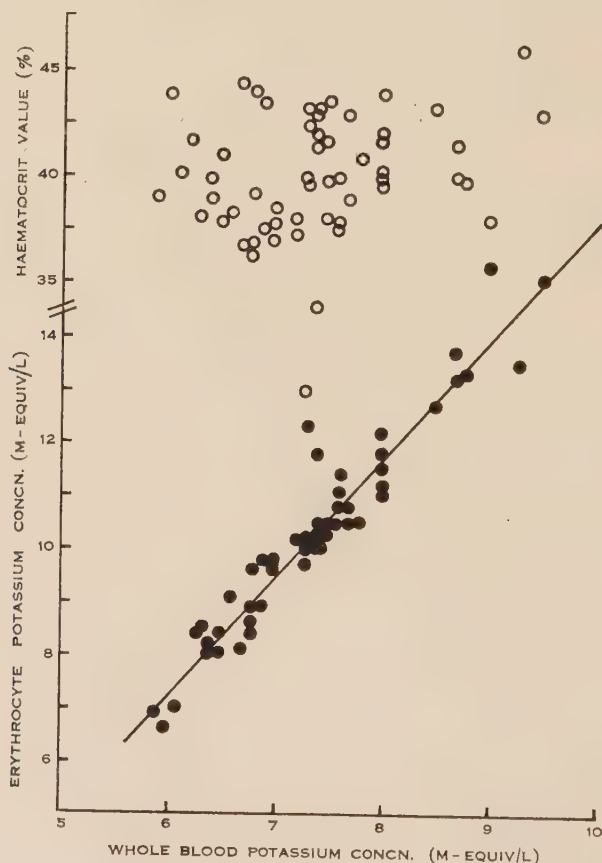


Fig. 1.—Correlation between whole blood potassium concentrations and erythrocyte potassium concentrations (●) or haematocrit value (○) in Merino ewes.

uniform type. At least 35 animals in any strain were sampled at a time and at any sampling at least two strains were bled so that between-strain variation could be assessed.

(b) *Bleeding*

Sheep were brought into the yards and allowed to settle down before a 5-ml blood sample was taken by jugular vein puncture using a 12-gauge needle and direct

withdrawal into a 7-ml screw-cap bottle containing heparin and which was free from both potassium and sodium. The first 1 ml of blood was not collected. 60–80 sheep were sampled per hour.

(c) Blood

A 1 : 400 dilution of each whole blood sample in deionized water was made within 2 hr of collection. The diluted samples were analysed for potassium and sodium using a flame photometer (King and Wootton 1956).

Because the difference in $[K_b^+]$ between *LK* and *HK* sheep is the result of erythrocyte differences it is possible to differentiate the erythrocyte types by using a whole blood analysis. The approximate $[K_e^+]$ of *LK* animals can be estimated from a whole blood analysis.

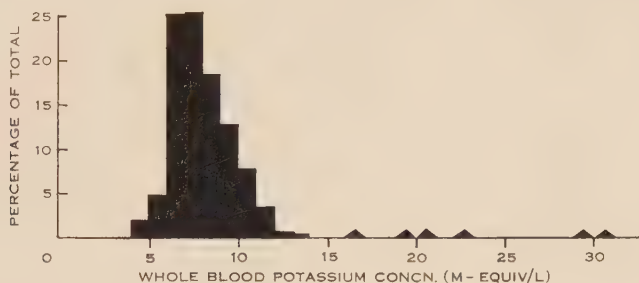


Fig. 2.—Percentage distribution of animals in a flock of Havilah Merino ewes according to the potassium concentration in the whole blood. ▲ Individual values.

Packed cell volume (P.C.V.) estimations were made after centrifugation at 1700*g* for 1 hr.

The type of haemoglobin in the erythrocytes was determined electrophoretically using Whatman No. 3MM paper, barbitone buffer at pH 8.6, and a 16-hr running time (Harris and Warren 1955).

III. RESULTS

A preliminary experiment on a flock of healthy Merino ewes showed that a linear relationship existed between $[K_b^+]$ and $[K_e^+]$ (Fig. 1), and that if $[K_e^+]$ is the only variable, the following relationships will hold: for $[K_b^+]$ values of 6, 8, 10, and 12 m-equiv/l, the corresponding $[K_e^+]$ values are 7.2, 11.7, 16.2, and 20.7 m-equiv/l respectively.

The results of an examination of 968 Havilah type fine-wool Merino ewes are shown in Figure 2. A mean $[K_b^+]$ value of 7.95 ± 0.07 m-equiv/l, representing a mean $[K_e^+]$ of approximately 11.5 m-equiv/l, was found. Four sheep (0.41%) had $[K_b^+]$ greater than 20 m-equiv/l and have been designated *HK* types, and the remainder *LK* types. The *HK* gene frequency $[(HK\%)^{\frac{1}{2}}/10]$ in this Havilah flock was therefore 0.064. Two of these four sheep had $[K_b^+]$ values of 29 and 30 m-equiv/l respectively, and were similar to the normal *HK* types found in British breeds in Great Britain,

TABLE 1
MEAN WHOLE BLOOD POTASSIUM CONCENTRATION, $[K_b^+]$, IN *LK* EWES IN FIVE MERINO STRAINS ON FIVE OCCASIONS
The *HK* gene frequency and derived mean erythrocyte potassium concentration ($[K_e^+]$) is also given

Sample* No.	No. of Ewes of each Strain Used and Mean $[K_b^+]$ Values (m-equiv/l)							
	Fine Non-Peppin		Medium Non-Peppin		Strong Non-Peppin		Medium Peppin A	
	No.	Mean \pm S.E.	No.	Mean \pm S.E.	No.	Mean \pm S.E.	No.	Mean \pm S.E.
1	35	7.61 \pm 0.25	35	8.11 \pm 0.26	35	9.89 \pm 0.40	36	8.27 \pm 0.31
2	49	8.18 \pm 0.17			57	9.72 \pm 0.27		
3	59	8.60 \pm 0.16			39	9.49 \pm 0.34		
4	54	7.92 \pm 0.22			34	10.13 \pm 0.40	90	8.54 \pm 0.16
5	31	8.19 \pm 0.18					98	8.42 \pm 0.07
Total	228	8.01 \pm 0.08	35	8.11 \pm 0.26	165	9.80 \pm 0.17	126	8.45 \pm 0.15
$[K_e^+]$ (m-equiv/l)		11.7		11.9		15.7		12.6
No. <i>HK</i> sheep (%)		0.0		0.0		2.42		0.0
<i>HK</i> gene frequency†		0.0		0.0		0.16		0.0

* Different animals were bled at each sampling period.

† i.e. $\sqrt{(HK\%)/10}$.

i.e. type $Ke\gamma$ (Evans 1957a). The other two HK animals, together with one with $[K_b^+]$ of 19 m-equiv/l would be $Ke\gamma$ type animals only if their haematocrit value were exceptionally low. They were more than likely type $Ke\beta$ or $Ke\delta$ which are relatively common in Middle East breeds (Kerr 1937) and have been shown to exist in fine-wool Merino sheep (Evans 1957a).

In a further experiment the mean $[K_b^+]$ in LK ewes of five Merino strains sampled on five occasions was determined. Results are given in Table 1. The mean value for the strong non-Peppin (SNP) strain was significantly higher than for the other strains examined ($P < 0.1$). There were differences among these strains and the mean

TABLE 2
MEAN WHOLE BLOOD POTASSIUM CONCENTRATION ($[K_b^+]$), AND THE DERIVED MEAN ERYTHROCYTE POTASSIUM CONCENTRATION ($[K_e^+]$) IN LK EWES COMPARED WITH LK RAMS SAMPLED AT THE SAME TIME

Strain	Ewes			Rams			Differences Significant at $P <$
	No.	Mean $[K_b^+]$ (m-equiv/l) \pm S.E.	Approx. $[K_e^+]$ (m-equiv/l)	No.	Mean $[K_b^+]$ (m-equiv/l) \pm S.E.	Approx. $[K_e^+]$ (m-equiv/l)	
FNP	35	7.61 ± 0.25	10.8	10	11.21 ± 0.52	18.9	0.1
MNP	35	8.11 ± 0.26	11.9	9	9.58 ± 0.65	15.2	5.0
SNP	35	9.89 ± 0.40	15.8	8	11.16 ± 0.85	18.6	n.s.
MPA	36	8.27 ± 0.31	12.2	9	10.91 ± 0.73	18.2	0.1
MPB	39	7.89 ± 0.25	11.4	8	10.27 ± 0.67	16.8	0.1
Total	180	8.34 ± 0.13		44	10.63 ± 0.30		0.1

concentration for both medium Peppin strains MPA and MPB was significantly higher at the 1 and the 5% levels respectively than for the fine non-Peppin strain (FNP). Results for the medium non-Peppin (MNP) are also given in Table 1.

The gene frequency for HK in four of the strains examined was 0.00. HK sheep were found only in the SNP strain (in 4 out of a total of 165) and the HK gene frequency was 0.16 (Table 1).

Rams from all five strains were also bled when the ewes were first sampled and the results are given in Table 2. The $[K_b^+]$ of the rams was higher in all strains. The overall difference between ewes and rams is significant at the 0.1% level. However, the variance within rams and within ewes is approximately the same.

The P.C.V. percentage of 108 4-year-old Havilah strain Merino ewes at pasture was determined during the investigation and a mean value of 32.1% was found.

The gene frequency of type *A* haemoglobin for the five strains is given in Table 3. No significant difference was found among the three non-Peppin strains but a significant difference between non-Peppin and Peppin strains was observed ($P < 0.1$).

IV. DISCUSSION

Only 8 out of a total of 1655 mature Merino sheep had $[K_b^+]$ greater than 20 m-equiv/l. These eight animals have been classified as *HK*, and therefore the overall gene frequency for *HK* in these flocks was 0.07. A similar frequency has been found in the English Leicester, but the great majority of British breeds have much higher frequencies (Evans, Harris, and Warren 1958a). The fact that 4 of the 8 *HK* sheep were found in the 165 SNP animals (gene frequency for *HK* = 0.16) suggests that the gene frequency for *HK* in fine-wool Merinos is exceptionally low (0.05).

TABLE 3
GENE FREQUENCY FOR HAEMOGLOBIN TYPE *A* IN THREE NON-PEPPIN AND TWO PEPPIN FLOCKS

Strain	No. of Sheep in each Flock	Haemoglobin Type			Gene Frequency* for Haemoglobin Type <i>A</i>
		<i>A</i>	<i>AB</i>	<i>B</i>	
Havilah	136	42	69	25	0.56
Fine non-Peppin	136	47	67	22	0.59
Strong non-Peppin	144	45	65	34	0.54
Medium Peppin A	22	0	8	14	0.18
Medium Peppin B	20	4	8	8	0.40

* Gene frequency for haemoglobin type *A* significantly higher in non-Peppin strains ($P < 0.1$).

† Mean values.

The Merino is quite unusual with respect to this feature when compared with the 35 British breeds which have been extensively studied. The fine-wool Merino is also distinctly different from all British breeds (again with the possible exception of the English Leicester (Evans and Mounib 1957)) in that it has a lower mean $[K_b^+]$ in *LK* animals than all other breeds of sheep so far examined. The mean $[K_e^+]$ of fine-wool Merino sheep of approximately 11.5 m-equiv/l found is similar to that found by Denton *et al.* (1951) and Harris, McDonald, and Williams (1952), and Evans (1957a). The *LK* ewes in the SNP strain showed significantly higher values for $[K_e^+]$ (approx. 15.7 m-equiv/l) than all the other strains.

Previous work Evans and Mounib (1957) has shown that a high mean $[K_b^+]$ for *LK* animals is associated with a high gene frequency for *HK*. The present data support this.

The histograms (Fig. 3) for *LK* types within a strain show a distinct and significant skewing with the tail towards *HK*. This result could be due to a number of factors, e.g. the result of a truncated distribution or of two populations of cells—

the population with the lower mean potassium concentration far outnumbering that with the higher concentration. Evans *et al.* (1956) have shown that the mean $[K_b^+]$ of known heterozygous animals in a flock is significantly higher than $[K_b^+]$ of the remaining *LK* animals (homozygous and heterozygous). They estimate that the difference between

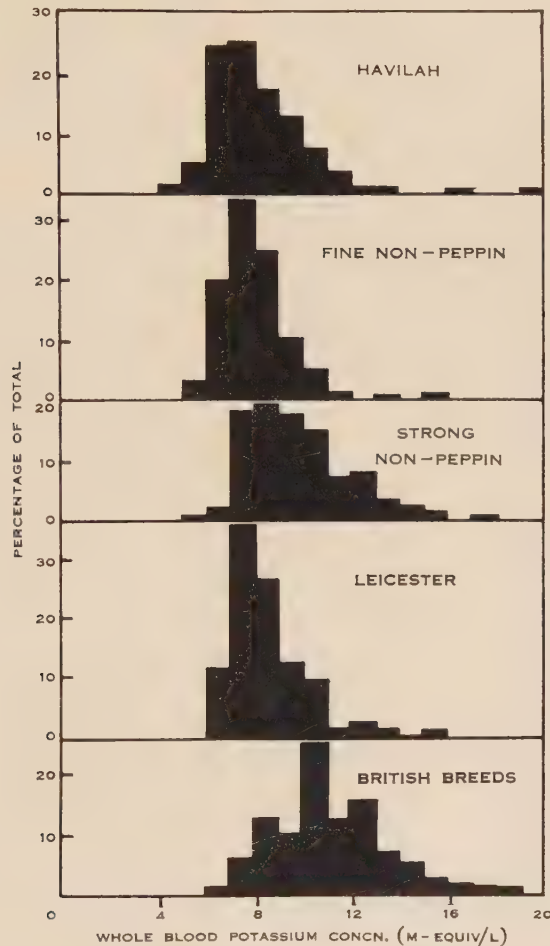


Fig. 3.—Percentage distribution of *LK* animals in three Merino strains compared with Leicester and other British breeds of sheep (see Evans and Mounib 1957) according to the whole blood potassium concentration.

homozygous and heterozygous animals would be 1.7 m-equiv/l whole blood. The difference in $[K_e^+]$ between the two groups would be of the order of 4.0 m-equiv/l erythrocytes. The possibility of at least two distinct groups in the *LK* phenotype has been discussed previously, particularly in relation to Romney Marsh and Merino sheep (Evans 1957a).

The haemoglobin type of an animal also has an effect on $[K_e^+]$. *LK* animals which have haemoglobin type *A* have a higher $[K_e^+]$ than those which have haemoglobin type *B* (Evans 1961).

In any animal in which $[K_b^+]$ is approximately 5 m-equiv/l, it must be assumed that plasma and erythrocyte concentrations of potassium are similar. In the Merino strains examined here $[K_b^+]$ approached this value ($[K_b^+]$ for Havilah flock = 7.95 m-equiv/l compared with approx. 12 m-equiv/l in Scottish Blackface sheep). Although animals with $[K_e^+]$ of between 5 and 6 m-equiv/l were not uncommon, only seven animals had $[K_b^+]$ between 4 and 5 m-equiv/l. This lower value of 5 m-equiv/l seems to be common to many breeds, e.g. British Hill, British Lowland, and Merino, and the only peculiarity of the Merino is that a larger proportion of the animals in this breed approach this apparently minimal value.

If the distribution in the *LK* Merino animals is a truncated distribution, it is then necessary to assume that a relatively large proportion of the animals in the Havilah flock are not represented (Fig. 2)—presumably not conceived, resorbed, or dead. A loss of this order would appear to be quite high even for a breed such as the Merino which is not noted for its high lambing percentages. Losses of the magnitude suggested by the data presented in this paper are unlikely to occur in most years. A truncated distribution also means that foetuses which as adults would have to maintain $[K_e^+]$ below that in the plasma would have to be envisaged. The absence of animals with $[K_b^+]$ below 5 m-equiv/l suggests that a mechanism of this nature, even if it were feasible, does not occur in adult sheep. A mechanism of this general nature could, however, exist in the foetuses which do not survive. It seems therefore that the distribution may represent a truncated normal curve and therefore be of interest in relation to lambing percentages, but that other explanations must also be considered.

The fact that all foetal erythrocytes appear to have a high $[K_e^+]$ (c. 115 m-equiv/l (Widdas 1954; Evans and Blunt 1961a)) must also be borne in mind. This high foetal concentration is found in the red blood cells of animals destined to become *LK* types as adults, the change from high to low potassium concentration occurring over the last 40 days of prenatal life and the first 3–4 months of postnatal life (Evans and Blunt 1961a).

If it is assumed that the distribution is a skewed and not a truncated distribution and that the red blood cells of animals at the lower limit (5 m-equiv/l) are in equilibrium with the plasma in which they are bathed, the postulation of an active process in relation to the concentration of potassium in the erythrocytes of these animals becomes unnecessary. It must be assumed that an active process is involved in the maintenance of the high potassium concentrations in the *HK* animals. It would then be reasonable to postulate that the difference between 5 m-equiv/l (low *LK*) and 85 m-equiv/l animals (*HK*) was the result of homozygosity for one or other of a pair of alleles controlling a mechanism for maintaining high intracellular potassium concentrations.

In any strain or breed where the *HK* gene frequency is exceptionally low (e.g. Havilah Merino, English Leicester) the mean $[K_e^+]$ in the *LK* animals would tend towards the plasma value because of the relatively small percentage of heterozygous

animals within the *LK* group. If the gene frequency for haemoglobin type *B* is high the tendency towards low $[K_e^+]$ would be accentuated.

If no dominance is assumed the heterozygous animal would have $[K_e^+]$ values half-way between those of the *LK* and *HK* animals (i.e. approx. 45 m-equiv/l erythrocytes), and the distribution would be trimodal with means of approximately 5, 45, and 85 m-equiv/l. It is known, however, that the *LK* gene is dominant (Evans and King 1955) and it is also known that this dominance is incomplete (Evans *et al.* 1956).

The observations referred to above were carried out on Scottish Blackface sheep and, although it is unlikely that the Merino is different, this possibility must not be overlooked particularly when Evans (1957*a*) has shown that this breed differs from British breeds with respect to the distribution of animals in the *LK* group.

From the experimental evidence reported here and by Evans (1957*a*), it seems necessary to postulate that the mean $[K_e^+]$ of a homozygous *LK* animal is between 10–12 m-equiv/l, and not 5 m-equiv/l, as would be suggested by a genetic system which resulted in the absence of a mechanism which produced high $[K_e^+]$. Although animals with $[K_e^+]$ lower than 10 m-equiv/l do undoubtedly exist (Evans 1957*a*) they must be considered to represent normal variation about a higher mean value which has been determined genetically, their lower value being the result of other secondary physiological or genetic factors. In relation to this it could be argued that homozygous *LK* animals which have $[K_e^+]$ much lower than this mean value might be at a disadvantage during any pathological or physiological state in which the plasma potassium concentration was markedly altered, and that this should be considered in relation to the lamb *in utero* (and therefore lambing percentages) as well as in the adult animal itself. The results presented here suggest that a $[K_e^+]$ of approximately 12 m-equiv/l is an advantage in a Merino in Australia. The relatively high gene frequency for haemoglobin type *A* as compared with other breeds with low gene frequencies for *HK* may be a compensatory mechanism. The recent work of Evans and Blunt (1961*b*) in which two British breeds of sheep were shown to change towards Merino-like gene frequencies for *HK* and haemoglobin type *A* after they had been established in Australia, supports this possibility. This approach to the problem of low lambing percentages, when considered along with the higher $[K_e^+]$ of rams as compared with ewes, and the possibility therefore that steroid hormones may modify the expression of the gene, and the known elaboration of androgenic hormones from the adrenal gland, may open a further approach to *in utero* losses, particularly when adverse conditions such as, for example, drought, prevail during pregnancy.

It seems reasonable to postulate therefore that *LK* Merino animals represent animals with $[K_e^+]$ varying about a mean value of approximately 11 m-equiv/l (homozygous) and a mean of at least 15 m-equiv/l (heterozygous), and that the gene which is partially dominant controls a mechanism for the maintenance of intracellular potassium concentrations at a level either approximately 6 m-equiv/l or approximately 80 m-equiv/l above that in the plasma.

The nature of the composition of the blood of the heterozygous animal is also of interest because the actual $[K_e^+]$ could be the result of a homogeneous population of cells with an intracellular mechanism controlling potassium concentration which

resulted in a mean concentration of approximately 15 m-equiv/l, or two populations of cells (with $[K_e^+]$ 11 and 85 m-equiv/l respectively) in such a ratio that a mean $[K_e^+]$ of approximately 15 m-equiv/l is maintained.

This latter possibility has been suggested by Joyce and Weatherall (1958) who showed that there is a fast- and a slow-moving fraction of cell potassium and sodium and that the size of the fast fraction is independent of the initial concentration of these electrolytes in the cell. The fast fraction is not peculiar to sheep cells (Clarkson and Maizels 1955; Joyce, Rayner, and Weatherall 1956). Joyce and Weatherall (1958) point out that it is unwarranted to assume a homogeneous population; but that "the variation in the size of the fast fraction is in better accord with the concept that it lies near the surface of all the cells than that it is located in a small number of special cells".

Tosteson and Hoffman (1958) have suggested that "cell cation content is regulated (a) by a pump which moves sodium ions out of the cell in exchange for an external potassium ion, and (b) by parallel diffusion leaks for both sodium and potassium. The parameter α is the ratio of the sodium to the potassium leak rate constant, while β is the ratio of pump to leak influx of K". In both *LK* and *HK* sheep cells the cation pump exchanged one sodium for one potassium but both α and β were much higher in cells from *HK* animals than in cells from *LK* animals.

It is impossible to decide between the two possibilities but it would appear at the moment more logical to assume a homogeneous cell population, rather than a mixture of *HK* and *LK* cells, in the heterozygous animal.

Differences in median corpuscular fragility between homozygous *HK* and homozygous *LK* cells have been demonstrated (Evans 1961) and can be interpreted to mean that *LK* cells are more spherocytic, and possibly more fragile, than *HK* cells (Dacie 1956). If this interpretation is valid then it should be possible to distinguish two populations of cells in the heterozygous animal if the cell population of a heterozygous animal is the result of a mixture of *HK* and *LK* cells, the *LK* far outnumbering the *HK*. No evidence for two populations of cells was seen in the small number of animals which were critically examined with this possibility in mind.

Present evidence suggests therefore that heterozygotes have one circulating erythrocyte type, and that these cells have a mechanism for maintaining the $[K_e^+]$ at approximately 15 m-equiv/l, and that the skewed distribution of the *LK* phenotype which is seen in breeds with a low gene frequency for *HK* is due to a small proportion of animals with this type of cell in the sample population. This tends to be supported by the apparently greater skewing of the SNP population compared with the FNP (Fig. 3).

As has already been mentioned, the significant difference in $[K_e^+]$ between rams and ewes is of considerable interest. It is also interesting that the difference between the mean *LK* $[K_b^+]$ between SNP ewes and the other strains of ewes is significant whereas the difference between SNP rams and other strains of rams is not significant. The difference could be a result of selection, in that different criteria may be used to select rams and ewes, or it could equally indicate that sex hormone levels, or factors controlled by them, influence the $[K_e^+]$ level.

The results obtained by Turner and Koch (1961) do not show a similar difference between ewes and rams. The different results obtained in this paper could be due to differences in the age of the animals involved or the fact that some of their male animals were wethers.

It could be argued that stud rams would have been more carefully selected and husbanded and that the improved husbandry could result in higher P.C.V. values and therefore higher mean $[K_b^+]$ values. The rams used in these experiments did not receive any special treatment, and husbandry would be an unlikely factor. The differences in P.C.V. values between ewes and rams would have to be very great (all other things being equal) to account for the differences in potassium concentration which were observed. Also Figure 1 shows that $[K_b^+]$ values show no definite relationship to haematocrit values, whereas a very significant correlation between $[K_b^+]$ and $[K_e^+]$ is demonstrated. It seems likely therefore that the sex of the animal *per se* affects the mean $[K_e^+]$ in Merino sheep.

When the whole blood data was being considered it was necessary to obtain some idea of the normal P.C.V. values in the Merinos in the area in which the experiment was carried out. A mean value of approximately 42% had been found for the Scottish Blackface breed, and it was therefore surprising to find that 4-year-old Havilah strain Merino ewes at pasture gave a mean value of 32.1% and that values under 30% were quite common. A group of 4-month-old lambs, hand-fed in pens, showed a mean haematocrit over 40%, however (Fig. 1).

The low P.C.V. value in the Havilah flock at pasture suggests that the flock as a whole might have been suffering from a subclinical anaemia. However, it could have been due to a very high gene frequency for sheep haemoglobin type *B*, since Mounib and Evans (1959) have shown that haemoglobin type has an effect on haematocrit percentage, haemoglobin type *A* animals having higher haematocrit percentages than haemoglobin type *B* animals. These workers were also able to demonstrate a higher dry matter percentage and specific gravity in the erythrocytes of haemoglobin type *B* animals than in type *A* animals. A mean haematocrit of 32% is, however, much lower than would be expected even if all the animals were type *B* haemoglobin. Evans, Harris, and Warren (1958*b*) have shown that the Merino is different to most British breeds of sheep in that the association of a high *HK* gene frequency with a high haemoglobin type *A* gene frequency, which exists for British breeds (Evans, Harris, and Warren 1958*a*), does not hold for the Merino, the Merino having a relatively high frequency of haemoglobin type *A* associated with a very low frequency for *HK*. It was unlikely therefore that the animals examined in the present experiment would be all haemoglobin type *B* and the relatively low mean haematocrit value was therefore even more interesting. A number of Merino sheep which had been used in this experiment were examined for haemoglobin type to check this point, and the frequencies given in Table 3 were found. These agree quite well with those previously found by Evans, Harris, and Warren (1958*b*), and indicate that the relatively low haematocrit values found in this experiment cannot be attributed solely to the haemoglobin type of the animals used. The relationships between low haematocrit values and raised erythrocyte potassium concentrations are under investigation in connection with an anaemia in Merino sheep which was discovered when the above observation was extended.

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A COMPARATIVE STUDY OF THE MONOLAYERS OF VARIOUS CEREAL PROTEINS AND OF WHEAT GLUTENS OF DIFFERING CHARACTERISTICS

By N. W. TSCHOEGL*

[Manuscript received January 6, 1961]

Summary

Surface films of the proteins extracted by anhydrous acid chloroethanol from strong wheat, weak wheat, rye, barley, oat, defatted oat, and carob germ flour were examined by monolayer techniques at the air/water and the oil/water interface. All proteins gave "ptygmatic" films showing very little differences in the II-A isotherms. They were clearly differentiated in their surface rheological characteristics. These studies indicated that the limiting surface viscosity at infinite time and the rate constant of the surface viscosity would be the most likely characteristics to differentiate between gluten films from different wheat flours. A comparative study of five different wheat glutens spread from dispersion in aqueous acid chloroethanol showed significant differences in the limiting surface viscosities but differences in the rate constants were not significant statistically.

I. INTRODUCTION

In two previous papers (Tschoegl and Alexander 1960a, 1960b) monolayer studies at the air/water (A/W) and the oil/water (O/W) interface were reported on films of wheat gluten, the elastic coherent protein complex obtained by washing out the starch and water-solubles from a wheat flour dough. The present paper extends these basic studies to a comparative study of monolayers of different cereal proteins and of different wheat glutens. The first part was carried out chiefly to obtain pointers which could be followed up in comparing the proteins from wheat flours of different baking quality and handling properties. Such differences in wheat flours are reflected, on an exaggerated scale, in similar differences among cereals. Of the commoner cereals only wheat and rye may be regarded as bread cereals. From barley and oats it is not ordinarily possible to obtain an aerated loaf of bread. The protein of the germ of the carob bean, *Ceratonia siliqua*, was included in this study since it is the only known non-wheat plant protein capable of forming a coherent gluten.

It is hoped that these investigations will eventually contribute to our understanding, in terms of gluten structure, of the reasons for differences in the baking quality and handling properties of different wheat flours. A possible practical application would be in the field of wheat breeding. Very small samples of test material are sufficient for examination by monolayer techniques. If a reliable correlation can be found between film properties and flour quality, the baking characteristics of a new wheat strain could be predicted from quite small samples.

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II. MATERIALS AND METHODS

Table 1 shows the analytical and physical testing data of the flours used. The procedures by which these flours were obtained and the analytical methods employed are described elsewhere (Tschoegl 1961). Defatted oat flour was prepared by extracting the oat flour twice for 5 hr with petroleum ether (boiling range 40–60°C).

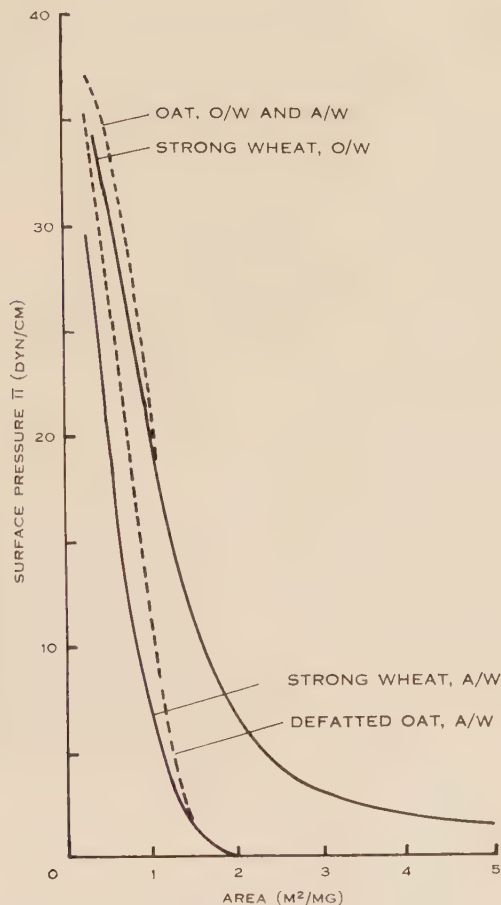


Fig. 1.—Surface pressure–area isotherms at pH 6.8 and $\mu = 0.1$.

The wheat flours were chosen according to “strength” (baking quality) and “balance” (extensibility of the dough) as determined on the Chopin alveograph. The strong, medium, and weak flours were “well-balanced”, while the harsh, medium, and extensible flours were all of medium strength.

The dispersions used in the comparative study of cereal proteins were prepared by direct extraction of the flours with a 0.1N solution of hydrogen chloride in carefully purified anhydrous 2-chloroethanol. The dispersions used in the com-

TABLE 1
ANALYTICAL AND PHYSICAL TESTING DATA OF FLOURS

	Strong Wheat	Weak Wheat	Medium Wheat	Harsh Wheat	Extensible Wheat	Rye	Barley	Oat	Defatted Oat	Carob Germ
Protein (% dry basis)	11.71	9.92	12.50	10.60	10.12	6.52	6.15	8.20	10.52	62.2
Moisture (%)	15.0	12.8	14.4	14.6	14.0	13.4	12.2	9.2	10.8	9.3
Ash (% dry basis)	0.55	0.53	0.48	0.57	0.46	0.38	0.59	0.74	—	—
Fat (% dry basis)	1.91	2.06	1.80	1.97	1.85	1.56	1.70	8.68	2.37	9.20
Fat/protein ratio	0.16	0.21	0.14	0.19	0.18	0.24	0.28	1.06	0.23	0.15
Alveogram "strength"	84	13	48	46	35	—	—	—	—	—
Alveogram "balance"	1.23	1.10	1.42	0.75	3.56	—	—	—	—	—

parative study of wheat glutens were prepared by dispersing the vacuum-dried glutens in a 0.01N solution of hydrochloric acid in aqueous 2-chloroethanol containing 30% water. The preparation of the glutens and the dispersants is described in detail elsewhere (Tschoegl 1961).

Films were spread at the interface between air or carbon tetrachloride and various aqueous substrates (buffers of different pH and ionic strength (μ)) from an all-glass "Aglar" syringe as detailed by Tschoegl and Alexander (1960a). Surface pressure measurements were carried out with two hanging plate surface balances, either in a Langmuir trough (A/W), or in a circular crystallizing dish (O/W). Surface viscosity and rigidity were determined using an oscillating needle torsion pendulum. Details of apparatus and methods are given in the two preceding papers (Tschoegl and Alexander 1960a, 1960b). All curves shown represent the average of at least two runs. The measurements were carried out at $25 \pm 0.5^\circ\text{C}$.

III. RESULTS

Surface pressure (II, dyn/cm)–area (A , m^2/mg) curves were obtained at the A/W interface at pH 6.8 (μ 0.02 and 0.1) and at the O/W interface at pH 6.8 and 2.1 (μ 0.1) from the extracts of strong and weak wheat flour, rye, barley, oat, defatted oat, and carob germ flour.

All the extracts behaved very similarly to gluten (Tschoegl and Alexander 1960a) in all essentials, showing great stability, high compressibility (about 0.05 cm/dyn at about $1.1 \text{ m}^2/\text{mg}$), hysteresis effects depending on the ionic strength of the substrate, an effect of pH on the expansion of the isotherms at areas above about $1.1 \text{ m}^2/\text{mg}$, and little effect of ionic strength on the isotherms. All compressibility–area curves also showed, although in varying degrees, the characteristic kink between 2.5 and $3.5 \text{ m}^2/\text{mg}$ at the O/W interface.

The wheat, rye, barley, and carob germ isotherms were virtually identical at the O/W interface and are represented in Figure 1 by the strong wheat isotherm. There was a little more variation at the A/W interface where the weak wheat, rye, barley, and carob germ isotherms fell slightly to the right of the strong wheat isotherm shown in Figure 1. The oat isotherm at the O/W interface departed from the others at areas below about $1.1 \text{ m}^2/\text{mg}$. At the A/W interface the behaviour of the oat isotherm was abnormal in that it was identical with that obtained at the O/W interface. The defatted oat flour extract, however, gave the A/W isotherm shown in Figure 1 while at the O/W interface the isotherm was identical with the undefatted oat isotherm. The undefatted oat flour had a much higher fat/protein ratio than the other samples (cf. Table 1). The softening point of the oat fat was much lower than that of the others. This oil could be expected to be spread along with the protein from the chloroethanol and the undefatted oat flour therefore appears to have been spread as a mixed film.

Since the surface viscosity (η_s , surface poise) and surface rigidity (G_s , dyn/cm) of wheat gluten had shown a marked dependence on the pH of the substrate (Tschoegl and Alexander 1960b) a study of this effect was made on the same extracts by measuring η_s and G_s as a function of the area at the O/W interface. These measurements were carried out at pH 6.8 and 2.1 at an ionic strength of 0.1.

Plots of η_s and G_s against area gave very similar pictures. Figure 2, therefore, shows plots of the absolute surface modulus (\bar{G}_s , dyn/cm), a combination of η_s and G_s (Tschoegl and Alexander 1960*b*), against area. In order of increasing modulus at the same area the films fall in the order: strong wheat, weak wheat, rye, barley, oat, carob. The pH of the substrate is seen to have an effect on \bar{G}_s in all cases, resulting in a shift of the curves obtained at pH 2.1 towards lower areas compared with the pH 6.8 curves. The magnitude of the shift, however, varies markedly with the type of protein studied. The effect is most marked with the proteins of the bread cereals, wheat and rye, and least marked with carob germ protein. The non-bread cereals, barley and oat, occupy a middle position.

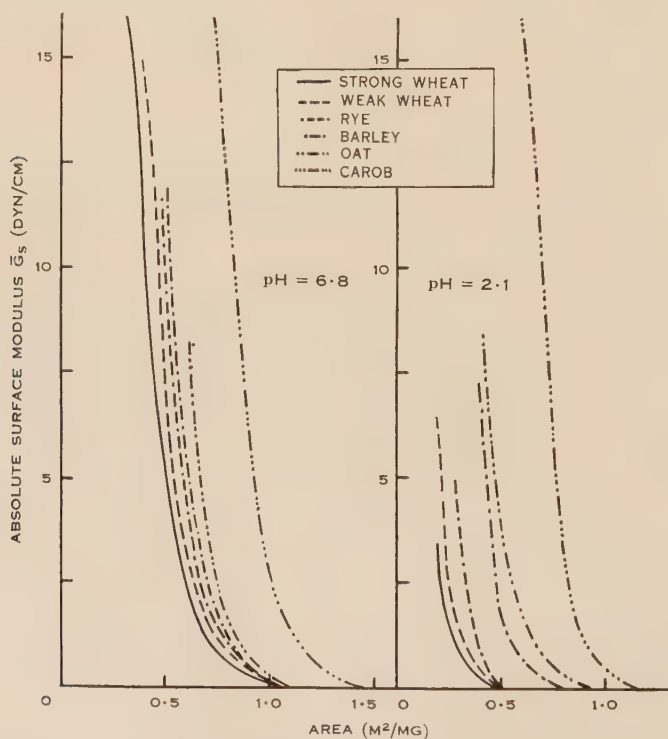


Fig. 2.—Absolute surface modulus \bar{G}_s as a function of the area at pH 6.8 (left) and 2.1 (right). O/W, $\mu = 0.1$.

It was shown previously that the surface viscosity of wheat gluten increases with time while the surface pressure remains essentially constant. If it is true that this increase reflects bond formation in the film (Tschoegl and Alexander 1960*b*) differences in the cohesiveness of the cereal “glutens” might possibly show up in this time dependence. Strong wheat and oat (defatted), representing the extreme placings of the cereals in the rank order referred to above, were therefore selected for a detailed study. The surface viscosities were measured over a range of pH values of the substrate at $\mu 0.02$. The areas at which the films were spread were chosen to yield

comparable limiting viscosities. The experimentally determined values were fitted by the method of "internal least squares" (Hartley 1948) to the relation (Tschöegl and Alexander 1960*b*):

$$\ln (\eta_{\infty} - \eta_t) / \eta_{\infty} = -K(t - t_0),$$

where η_{∞} is the limiting surface viscosity at infinite time, η_t is the instantaneous surface viscosity, K is the rate constant, t the time, and t_0 the time at which the surface viscosity is zero.

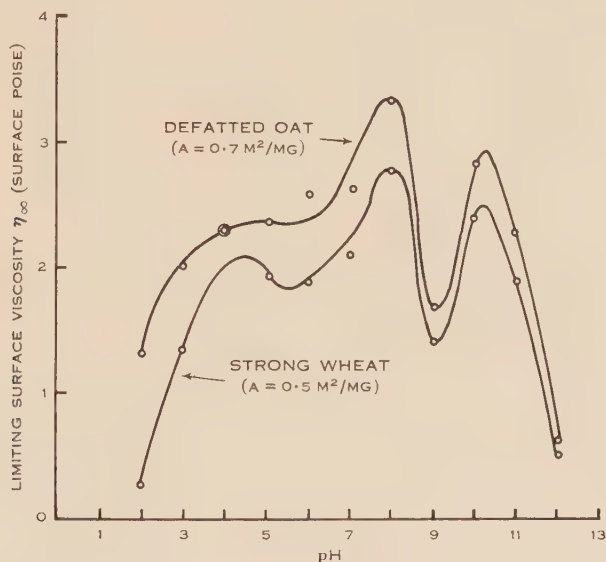


Fig. 3.—Limiting surface viscosity as a function of pH. O/W, $\mu = 0.02$.

Figure 3 shows a plot of η_{∞} against pH. This plot again reveals a maximum between about pH 7 and 8. There is a minimum around pH 9 and an indication of a minimum around pH 5.5. These curves are therefore similar to the plots of the area at 1 dyn/cm against pH, obtained from surface pressure measurements on gluten films (Tschöegl and Alexander 1960*a*). Below pH 4 the limiting surface viscosity of the strong wheat films dropped more sharply than the limiting surface viscosity of the oat films.

The plot of K against pH is shown in Figure 4. A maximum appears again between about pH 7 and 8 but there is no indication of any minima around pH 5.5 or 9. At comparable limiting viscosities the rate constants of the oat films are considerably higher than those of the wheat films.

The foregoing work on cereal protein monolayers had shown that the most fruitful approach to a comparative monolayer study of wheat gluteins of differing characteristics might lie in the examination of the limiting viscosities and the rate constants near the isoelectric point. An examination of the chemical and physical changes occurring in wheat gluten on dispersion in anhydrous and aqueous acid

chloroethanol (Tschoegl 1961) had shown that partial esterification of free carboxyl groups and a slight loss of amide nitrogen occurs when gluten is dispersed in a 0.1N solution of hydrogen chloride in anhydrous chloroethanol while this does not occur in a 0.01N solution of hydrochloric acid in chloroethanol containing 30% water. The isoelectric point of gluten films spread from the latter dispersions was found to be 6.7 (Tschoegl 1961) while surface viscosity measurements on gluten films spread from anhydrous acid chloroethanol yielded an isoelectric point of 7.5 (Tschoegl and Alexander 1960*b*). Aqueous dispersions gave lower surface viscosities than anhydrous dispersions but in all other essential respects gluten films spread from these two different dispersants seemed to be identical.

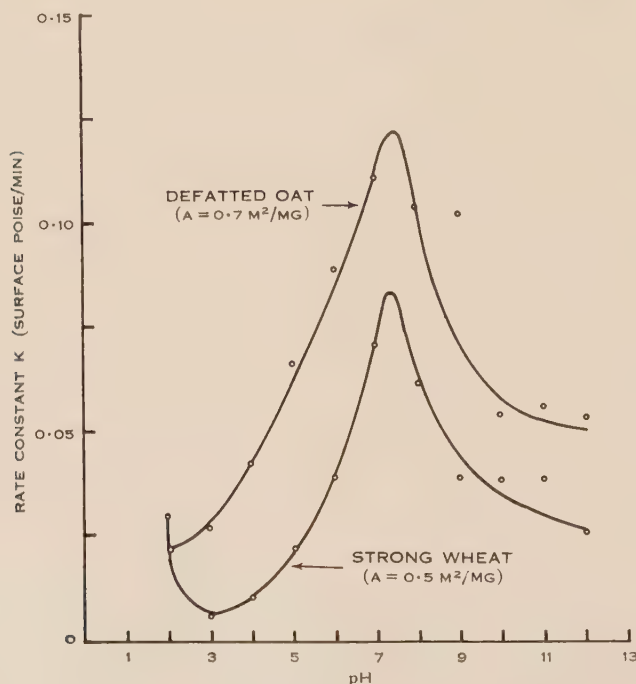


Fig. 4.—Rate constant of the surface viscosity as a function of pH. O/W, $\mu = 0.02$.

The glutes obtained from the five different wheat flours (Table 1) were therefore spread at the O/W interface from aqueous acid chloroethanol at pH 6.4, $\mu 0.02$, at an area of 0.4 m²/mg and the surface viscosities were measured as a function of time. Three curves were obtained from each dispersion and the limiting viscosities and rate constants were calculated individually and for the average curves. The results are shown in Tables 2 and 3.

Analysis of variance showed that the limiting viscosities were significantly different at the 1% level. The difference required for two means to be different at the 5% level was 0.33. The rate constants were not significantly different.

IV. DISCUSSION

Tschoegl and Alexander (1960a) ascribed the great stability and high compressibility of gluten films to the formation of "ptygmatic", or "folded", films at an interface and suggested that the required internal cohesion arose chiefly from the presence of an unusual amount of glutamine side-chains bearing the potentially

TABLE 2
LIMITING SURFACE VISCOSITY (SURFACE POISE) OF GLUTENS OBTAINED FROM FIVE WHEAT FLOURS

Replicate No.	Strong Wheat	Weak Wheat	Medium Wheat	Harsh Wheat	Extensible Wheat
1	1.08	1.45	1.29	1.87	1.21
2	0.81	1.42	1.26	2.31	1.82
3	0.92	1.87	1.27	2.02	1.35
Mean	0.94	1.58	1.27	2.06	1.46
S.D.	0.136	0.252	0.016	0.224	0.320
Average	0.93	1.40	1.29	2.05	1.44

strong hydrogen bond-forming amide ($-\text{CONH}_2$) end-group (Orgel 1959). Since a high content of glutamine residues is a common feature of the cereal proteins (Bourdett 1956) and the carob germ protein (Rice and Ramstad 1950), the fact that all the films investigated in this work showed the characteristics of ptygmatic films

TABLE 3
RATE CONSTANTS (SURFACE POISE/MIN) OF GLUTENS OBTAINED FROM FIVE WHEAT FLOURS

Replicate No.	Strong Wheat	Weak Wheat	Medium Wheat	Harsh Wheat	Extensible Wheat
1	0.0861	0.0525	0.0495	0.0328	0.0412
2	0.0563	0.0543	0.0365	0.0234	0.0288
3	0.0348	0.0568	0.0717	0.0314	0.0503
Mean	0.0590	0.0546	0.0526	0.0292	0.0401
S.D.	0.0258	0.0022	0.0178	0.0051	0.0108
Average	0.0552	0.0707	0.0436	0.0288	0.0396

lends support to this view. The II-A isotherms showed remarkable similarity at the O/W interface (cf. Fig. 1). The slight differences at the A/W interface could be due to differences in van der Waals cohesion. These would be expected to disappear at the O/W interface.

While surface pressure measurements thus did not clearly differentiate between cereal proteins, measurements of surface viscoelasticity produced marked differences.

At the same surface concentration (area), the absolute surface modulus increased in the rank order: strong wheat, weak wheat, rye, barley, oat, carob. Furthermore, the effect of pH on the absolute surface modulus clearly differentiated between the bread cereals and the non-bread cereals (Fig. 2). The rank order referred to above is the same in which the quality and cohesiveness of their "glutens" would place the cereal proteins (Cunningham, Geddes, and Anderson 1955). No gluten could be washed from the carob germ flour used in this study and therefore the carob germ protein also seems to fit into the trend shown by the cereal proteins. This is a little surprising as carob germ protein has been claimed by Rice and Ramstad (1950) to form a cohesive gluten. The preparative method used in this study (Tschoegl 1961) differed, however, from Rice and Ramstad's.

No satisfactory explanation can be offered at present for the differences in the influence of pH on surface viscoelasticity. Under the circumstances in which the measurements presented in Figure 2 were carried out, differences in the time dependence of the surface viscoelastic properties could be partly responsible. However, Figure 3 clearly shows the *limiting* surface viscosity at pH 2.1 of the oat protein film also to be higher than that of the strong wheat protein film.

More work would also be needed to explain the differences in the rate with which the limiting surface viscosities are reached in oat and wheat protein films. Differences in the rate constants of the five different glutens were not statistically significant. This is undoubtedly due in part to poor reproducibility.

Bulk viscosity measurements on gluten dispersions have not shown satisfactory correlation with baking strength although the sedimentation of flour suspensions in dilute lactic acid (Zeleny 1947) is said to afford a means for quick grading of wheat. Udy (1953) found that dilute acetic acid dispersions of different glutens gave different relative viscosities but he did not attempt any correlation with baking quality. While earlier experiments by Rose and Cook (1935), Harris and Johnson (1940), and Geerdes and Harris (1952) had indicated that "stronger" glutens might yield higher viscosities in sodium salicylate dispersions, Udy (1953) showed the relative viscosities of such dispersions to be independent of gluten quality.

For the five glutens examined here, the correlation coefficient between limiting surface viscosity and alveogram "strength" as a measure of baking quality was -0.566 which is not significant with only three degrees of freedom. Without the harsh gluten, however, the correlation would have been -0.991 which is significant even with only two degrees of freedom. A greater number of more precise measurements is clearly needed before correlation of film viscosity and baking quality could definitely be established or dismissed.

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THE QUANTITY OF WATER IN THE CELL WALL AND ITS SIGNIFICANCE

By D. F. GAFF* and D. J. CARR†

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Summary

Cell wall preparations from the leaves of *Eucalyptus globulus* Labill. may absorb water equal to approximately 150% of their dry weight.

The proportion of cell wall in the leaves of this species was estimated by three different methods, and the maximum water in the cell wall was calculated to constitute approximately 40% of the water content of the leaf at full turgor.

The implications of the large fraction of water in the cell wall are discussed. It is proposed that the cell wall, not the protoplast, acts as the main pathway for extrafascicular movement of water, and that in the leaf the cell wall water operates as a buffer against loss of water from the protoplast. The buffering capacity of the water in the wall may be a factor in the "hardening off" of plants to drought.

I. INTRODUCTION

The view that the cell walls of living cells are moist is firmly established in the literature, e.g. Crafts, Currier, and Stocking (1949), Kramer (1955), Stocking (1956). The general acceptance of the osmotic theory of the water relations of plant cells, however, has focused attention on the importance of the water enclosed by cell membranes. This has served to distract attention from the water in the cell wall. In recent years, however, it has been shown that the apparent free space (A.F.S.) forms an appreciable proportion of the volume of plant tissues. The A.F.S. is thought to be largely or entirely in the cell wall (Levitt 1957; Dainty and Hope 1959*b*), and its magnitude suggests that the water content of the cell wall may be high. For example, the A.F.S. of wheat and barley roots is of the order of 25% (Butler 1953; Epstein 1955). Black *et al.* (1960) reported that the A.F.S. of bacterial spores is as high as 40% and calculated that the cell wall represented a similar proportion of the cell. Work by G. W. Scott *et al.* (1957) indicates an A.F.S. of 90% for *Ulva lactuca*, the cell walls of which are thick and gelatinous. Data are presented in this paper to show that significant quantities of water are held in the cell wall of leaves of *Eucalyptus globulus* Labill., and to support the view that such water may play an important role in the water relations of plants.

II. MATERIAL AND METHODS

Homogeneous cell wall material was prepared from the fully expanded juvenile leaves of *E. globulus*. All leaves were taken from four saplings of similar age (5 years).

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(a) Preparation of Cell Wall Material

The midribs were removed and the laminae (in which veins are not prominent) were frozen with dry ice and ground to a fine powder by hand in a mortar and pestle. The ground leaf tissue was mixed with cold water, centrifuged at 3000 r.p.m. for 20 min at 2°C, and the residue again washed with cold water. Ethanolamine was added to the residue and the mixture ground in a top-drive homogenizer and then centrifuged. This procedure removes cytoplasm from cell walls (F. M. Scott *et al.* 1956) and presumably removes any protein constituent present in the wall.

The residue was subjected to this treatment repeatedly until, on centrifuging, the supernatant ethanolamine was light in colour. The residue was then homogenized in ethanolamine with a Potter-Elvehjem pestle homogenizer. The homogenate was left at 90°C for 4–5 hr and centrifuged. The residue was washed with cold water and three times with acetone, when there was only a faint coloration of the liquid.

Finally the moist residue was poured into shallow watch-glasses and allowed to dry in air to a flesh-coloured “paper” about 1 mm in thickness.

The total nitrogen of the preparation was determined by microKjeldahl techniques and nesslerization. Distillation of ammonia was performed using the apparatus described by Archibald (1943). The protein content was estimated using a factor (wt. of protein/wt. of nitrogen) = 6 (Chibnall 1939).

(b) Drying and Storage of the Cell Wall Material

Although it was desirable to refer water contents of the preparation to the dry weight, oven drying might have modified the hygroscopic properties (Wise and Jahn 1952). Consequently drying was performed at room temperatures by means of desiccators containing a saturated solution of lithium chloride in contact with an excess of the solid salt at 21°C. The cell wall preparation was supported by wire gauze about 2 cm above the surface of the solution. The saturated solution of lithium chloride, at 20°C, maintains a relative humidity of 15% (cf. 32% for relative humidity of air over a saturated solution of calcium chloride). This is sufficiently low to avoid growth of fungi on the cell wall preparation during storage in the desiccator.

Preliminary experiments showed that water-saturated cell wall preparations reached a constant weight in these desiccators within 24 hr (2 days were usually employed), and that the ratio of the “desiccated weight” so obtained to the “oven dry weight” (90°C for 2 days) was, on the average, 1.05.

(c) Uptake of Water Vapour by the Dry Cell Wall Preparation

Samples of the cell wall preparation were taken from storage in the desiccators, weighed, and placed in equilibration chambers containing water or sucrose solutions of various concentrations. Although the data for sucrose were obtained for other purposes, they are included here to support the results for water.

The equilibration chambers were glass phials 2 by 1 in., half-filled with filter paper saturated with solution. The cell wall preparation (approx. 1.5 by 1 cm) was supported on a tared glass plate resting on stainless steel gauze supported by the

wet filter paper (Fig. 1). The closed chambers were submerged in a stirred water-bath in which the temperature was controlled at $26 \pm 0.02^\circ\text{C}$.

Periodically each sample was slid off its glass plate into a tared weighing jar, weighed, and replaced in its equilibration chamber, or into a fresh chamber containing solution of the same concentration.

(d) *Loss of Water Vapour from the Moist Cell Wall Preparation*

Six samples of the cell wall preparation were removed from storage in the lithium chloride desiccators, and were placed in cold water in petri dishes. On contact with the water, the paper-like cell wall preparation expanded, at the same time becoming soft and difficult to handle. After 2 hr in water, the samples were manoeuvred on to small glass supports of known weight and were removed from

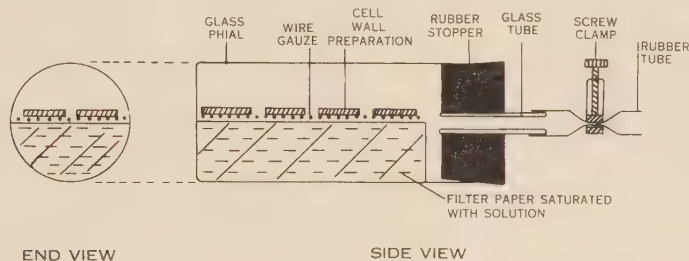


Fig. 1.—Apparatus used as equilibration chamber.

the water. The glass support and the cell wall material were carefully placed on a pad of filter paper with the support uppermost. When the circle of water on the filter paper ceased expanding, the paper was carefully peeled away from the surface of the cell wall preparation. During these manipulations loss of cell wall material was unavoidable. Nevertheless, the bulk of the sample of the cell wall preparation remained intact on the support.

Each sample, together with the glass support, was weighed in a tared weighing jar and then placed in an equilibration phial over sucrose solutions at 26°C ranging in concentration from 0 to 1.0M. The samples were reweighed at various intervals of time. Fresh equilibration phials and solutions were substituted periodically in order to avoid appreciable dilution of the solution.

The samples of cell wall material were left for 2 days in lithium chloride desiccators and then reweighed.

III. RESULTS

Tests with iodine solution showed that the cell wall preparation contained no starch. The leaves used contained 14% protein (estimated as a percentage of the oven dry weight) but no nitrogen was detected in the cell wall preparation. The removal of all nitrogenous materials from the cell wall preparation means that any such materials which may form an integral part of the cell wall are not represented

in the preparation. Opinions differ on the degree of penetration of the cell wall by the protoplast of the plant cell and, in view of the prevailing uncertainty on this point, it is perhaps preferable to confine this study of the hygroscopicity of the cell wall to its non-nitrogenous fractions.

(a) *Sorption by Dry Cell Wall Material*

The results are given in Table 1, where the weights of each sample have been corrected to an initial "desiccated weight" of 100 mg. Some of these data are plotted in Figure 2.

TABLE 1
SORPTION BY CELL WALL MATERIAL

Weights of samples corrected to an initial dry weight of 100 mg. The times indicated in brackets refer to the samples over 0.2M, 0.6M, and 0.8M sucrose

Time (hr)	Concentration of Sucrose in Equilibration Chamber				
	0 (water control)	0.2M (5 atm)	0.4M (11 atm)	0.6M (18 atm)	0.8M (26 atm)
0	100.0	100.0	100.0	100.0	100.0
5 (4.5)	141.7	147.4	142.1	140.8	133.3
21.5 (21)	181.8	182.8	172.9	165.7	151.0
29.5 (28.5)	190.1	188.2	179.8	166.9	152.3
45.7	201.8	—	193.1	—	—
53.5 (52.0)	208.6	209.8	198.6	175.8	158.7
70	217.7	—	202.3	—	—
75.5	220.1	—	204.9	—	—
94	224.5	—	207.8	—	—
(118.5)	—	239.4	—	178.9	159.1
166	259.4	—	223.6	—	—
Maximum water content (% dry wt.)	159	139	124	79	59

It is clear that the cell wall possesses marked hygroscopic properties. The dry cell wall can take up more than its own weight of water vapour. As is the case in live leaf disks (Carr and Gaff 1959), the cell wall material does not attain a virtual equilibrium in a short time. There is a continuous progress towards equilibrium at a continually decreasing rate. The data show that the cell wall material may absorb water vapour equal to approximately 150% of the dry weight. This value may be lower than the water content which may obtain in the cell wall in the intact leaf, where, in addition to water held by hygroscopic forces, there would be some water held in spaces in the cell wall. Consequently, desorption from wet wall material might give a greater value for the retention of water in the wall.

(b) Desorption of Wet Cell Wall Material

The water content of the samples after imbibing water for 2 hr is quite variable but averages about eight times the "desiccated weight" of the sample (see Fig. 3). It seems probable that most of the water is present as free water occupying the spaces between the cell wall fragments. In the equilibration chambers water is lost from the samples at a rate dependent on the difference in the water activity of the sample and of the sucrose solution. The rate of water loss, therefore, remains constant until all the free water has evaporated. Further water loss is at the expense of water held by imbibitional forces. Water loss at this latter stage increases the diffusion pressure deficit of the cell wall material, and results in turn in a reduced

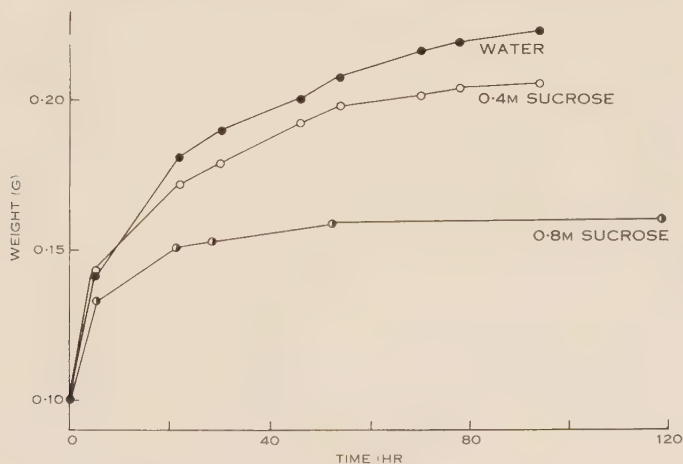


Fig. 2.—Time course of water uptake by desiccated cell wall preparations in equilibration phials containing water, 0.4M, and 0.8M sucrose. The weights of the samples have been corrected to a desiccated weight of 100 mg.

rate of water loss. The exponential loss indicative of this situation is most clearly suggested by the data for 1.0M sucrose where the loss of bound water is greatest.

The change from a constant to an exponential rate of water loss appears to occur at a water content of about 150% of the dry weight. This supports the conclusion that the maximum amount of water held in the wall is equal to approximately 150% of the dry weight of the wall material.

(c) Proportion of Cell Wall Material in the Leaf of E. globulus

Owing to the chemical complexity of the cell wall and the cell contents, it is difficult to obtain an accurate estimate of the proportion of cell wall material in a leaf even by using elaborate techniques. Three different methods were employed to obtain such an estimate.

(i) *Estimate 1.*—Freshly picked leaves, from which the midribs had been removed, were frozen with dry ice and ground to a powder. A sample of the powdered leaf was weighed in a tared, stoppered, weighing jar after the jar and its contents

had reached room temperature. The dry weight of the sample was determined after 2 days in an oven at 90°C.

The remainder of the powdered leaf was also weighed at room temperature, and transferred to a beaker containing distilled water. The mixture was boiled gently for 12 hr, then cooled, and centrifuged for 20 min at 3500 r.p.m. in tared "Nylex" tubes.

The residue was twice washed with distilled water and centrifuged. Finally, the residue was desiccated in the tubes at 90°C for 2 days and reweighed.

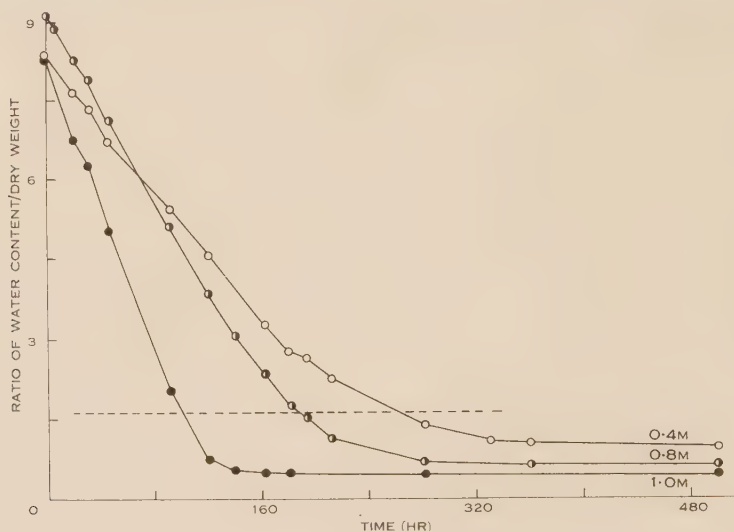


Fig. 3.—Time course of water loss from moist cell wall preparations in equilibration phials containing 0.4M, 0.8M, and 1.0M sucrose. The dotted line parallel to the abscissa represents approximately the water content at which the cell wall material is fully imbibed.

The nitrogen content of a sample of the dried residue was determined by microKjeldahl techniques and nesslerization. Protein was estimated to compose 11.4% of the dry weight of the residue. The remainder of the residue, assumed to be mainly cell wall material, amounted to 31.7% of the dry weight of the leaf tissue, i.e. 13% of the fresh weight.

The treatment of the ground leaf tissue with boiling water, in addition to removing the non-protein materials contained in the protoplast, would remove most of the hemicelluloses and pectins from the cell wall. The estimate given here for the amount of cell wall material must, therefore, be regarded as a minimum value.

(ii) *Estimate 2.*—A freshly picked leaf was folded along its midrib and corresponding areas were cut from the two halves of the leaf. The fresh weight of each sample was recorded. One sample was crushed between two sheets of blotting-paper, under a pressure of 2500 lb/sq. in. The crushed leaf was soaked in water for a few minutes and crushed once again between fresh blotting-papers. The crushed leaf was weighed and, together with the uncrushed sample of leaf, was dried for 2 days

at 90°C. The dry weights of the samples were recorded, and the proportion of cell wall material in the leaf was calculated.

Microscopic examination of leaf material crushed in this way showed that the vacuoles of the cells had been eliminated, but that the protoplasm remained in the cell cavities. A correction was therefore applied for the weight of protein remaining. On the basis of previous experiments, the weight of protein was taken to be equal to 14% of the dry weight of the uncrushed leaf. In this way the dry weight of the cell wall material was estimated to constitute 69% of the total dry weight of the leaf, i.e. approximately 27% of the fresh weight. The water content of the crushed leaf was 52% of the initial water content.

TABLE 2
DISTRIBUTION OF TISSUES AND CELL WALL IN THE LEAF

Tissue	Cell Volume as Percentage of Total Volume of Leaf	Cell Wall Volume as Percentage of Volume of Cell	Cell Wall in Tissue as Percentage of Total Volume of Leaf
Spongy mesophyll	43	12	5.2
Palisade			
Xylem			
Phloem			
Parenchyma sheath	4	8	0.3
Sclerenchyma	1	91	0.9
Collenchyma	4	35	1.4
Epidermis	16	19	3.0
Total	74*		12.7

* Intercellular spaces = 24%; oil in glands = 2%.

(iii) *Estimate 3.*—Microtome sections of the leaves were mounted in Canada balsam and measurements were made of images of the sections projected on to a sheet of tracing paper supported on glass. The areas occupied by the various tissues of the leaf were outlined on the paper for 16 microscope fields (objective $\times 45$, eyepiece $\times 12$, diameter of field *c.* 0.2 mm).

The paper was cut, sorted according to the type of tissue outlined, and weighed. The weights were taken to be a measure of the relative volumes of the component tissues of the leaf.

This technique was employed to estimate the proportion of cell wall in tissues with large cells and thick walls (xylem, collenchyma, sclerenchyma). In the other tissues the proportion of cell wall was estimated from means of measurements of the dimensions of a number of cells. The standard errors of the mean length, depth, and breadth of the cells were 3% or less of the mean, those of the wall thickness 7%. Calculations were based on cylinders, spheres, or rectangular prisms according to which of these geometrical shapes the form of the average cell most resembled. The results are given in Table 2.

The volume of the dehydrated cell walls constitute $[(12.7/74) \times 100]$, i.e. 17% of the total cell volume of the leaf. In further calculations it is assumed that the dry cell wall has the same relative density as amorphous cellulose (i.e. 1.48), and that the relative density of the protoplast is approximately that of 1M sucrose (which has an osmotic pressure equal to the mean osmotic pressure of the tissue at incipient plasmolysis). Then, bearing in mind that the cell wall imbibes water equal to 150% of its dry weight, we may estimate that the dry weight of the cell wall is equivalent to approximately 16% of the fresh weight of the lamina.

IV. DISCUSSION

(a) Water Content of the Cell Wall

The three separate estimates of the dry cell wall content of the lamina are 13, 27, and 16% of the fresh weight of the lamina. The difference between the first two estimates is probably due mainly to loss of pectins and hemicelluloses from the cell wall during boiling. Bishop, Bayley, and Setterfield (1958) report that, on a dry weight basis, at least 51% of the cell walls of the parenchyma of *Avena* coleoptiles is a mixture of hemicelluloses. It is not unreasonable, therefore, to expect leaf parenchyma cells to contain some hemicelluloses.

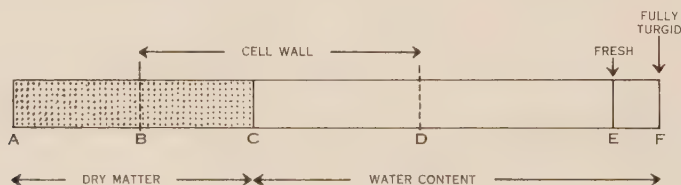


Fig. 4.—Relative quantities of dry matter and water in the leaf of *E. globulus*. AC, dry weight of leaf; BC, dry weight of cell wall material; CE, water content of a freshly picked leaf; CF, water content of a fully turgid leaf; CD, water content of the fully imbibed cell walls.

In addition, the presence of appreciable amounts of starch in the plastids would inflate the first and second estimates, despite the fact that the leaves were picked during the morning, to reduce the importance of this source of error.

For the purposes of estimating the quantity of water in the cell walls we take the mean of these three estimates, i.e. the dry weight of the wall (BC in Fig. 4) is 0.19 times the fresh weight of the lamina (AE). Since the water content of the fully imbibed wall (CD) is 1.5 times the dry weight of the wall (BC), the water content of the wall (CD) is 0.28 times the fresh weight of the leaf (AE). This is equivalent to approximately 0.4 times the water content at full turgor (CF).

A number of papers may be cited in support of the estimate of the hygroscopicity of the cell wall. Data demonstrating sorption and desorption of water vapour by various types of wood have been summarized by Stamm (1952). Jute absorbs water up to 34% (Pfuhl 1888), and manilla hemp and piassaba fibres take up water amounting to nearly 50% of the dry weight (Wiesner 1921). Cohn (1892) recalculated data

of other authors and obtained values for the water content of lignified tissues ranging from 31% of the tissue dry weight in *Pinus sylvestris* wood to 59% in oak sapwood, and reaching 120% in *Juncus* phloem fibres.

Christensen and Kelsey (1958) have studied the sorption of water vapour by the major components of the wood of *Eucalyptus regnans*. Various methods of extraction were used, and the hygroscopicity of the components was found to vary according to the extraction technique employed. Lignin can take up water equivalent to approximately 25% of its dry weight; hemicellulose takes up 75–100%, and holo-cellulose 33–52%. As might be expected then, lignified cell walls are less hygroscopic than other walls. For example, drying in air reduces the volume of phloem walls in potato stolons by 50% (Crafts 1931) and shrinks cambium cell walls to one-third their original thickness (Preston and Wardrop 1949). Hansteen-Cranner (1914) reported that preparations of the cell wall material of the pith from turnip petioles take up water equal to 170% of the dry weight of the wall material.

Cohn (1892) found that in four different species the water content of collenchyma cell walls was as high as 165–245% of the wall dry weight.

Hansteen-Cranner's data for pith and Cohn's data for collenchyma are comparable with the value obtained for the cell wall preparation from leaves of *E. globulus*.

Scarcely any published data are available on the hygroscopic properties of the cell walls of leaf tissue. One would expect that the hygroscopicity of this poorly lignified material would be considerable. Hartel (1951) investigated the swelling capacity of oven-dried leaves and concluded that it was due mostly to the cell wall material.

The high proportion of cell wall water in leaves of *E. globulus* is associated with a relatively high percentage dry weight of leaf (41% of the fresh weight), but this value is not exceptionally high for eucalypts (Blagoveshchensky and Bogracheva 1955). It is probable that similar amounts of cell wall water occur in the leaves of many plants.

(b) *Water Movement in the Mesophyll*

It has been pointed out by van den Honert (1948) that, in comparison with other resistances to water movement in the plant, the resistance offered by the protoplast is very large. Consideration of this, and of the large proportion of water in the cell wall, has led us to the view that extrafascicular water movement occurs mainly through the cell wall, and involves movement of capillary-condensed water. That is, the protoplast may not lie on the direct path of the transpiration stream.

The correctness of this view depends on whether or not the resistance to water movement of the path via the protoplasts in the leaf is greater than that via cell walls. It is quite clear from permeability studies that the protoplast presents a considerable resistance to the movement of water. Values for the water permeability of cells have been assembled by Bennet-Clark (1959) and range from 0.02 to $1 \mu \text{ min}^{-1} \text{ atm}^{-1}$, although values as high as $18 \mu \text{ min}^{-1} \text{ atm}^{-1}$ have been reported for *Nitella*. Mercer and Clark* found that the permeability of isolated tonoplasts plus vacuoles was up to 10 times the permeability of isolated protoplasts.

* Reported in Dainty and Hope (1959a).

Information on the relative resistances of the protoplast and the cell wall is meagre. The clearest demonstration of the lower resistance of the cell wall is presented by Levitt, Scarth, and Gibbs (1936), who found that the water permeability of free protoplasts of onion epidermis was the same as that of protoplasts enclosed in their cell walls. Wartiovaara (1944) reported that the permeability of isolated protoplasts of *Tolypellopsis* to deuterium hydroxide was 1.6 cm/hr compared with 1.04 cm/hr for intact cells. In considering this and other experiments in which isotopically labelled water was employed, the recent discovery by Hübner (1960) that extremely rapid loss of labelled water to the atmosphere occurs during manipulation of tissue segments, must be borne in mind. This would invalidate absolute values such as those of Wartiovaara. Nevertheless, comparative studies would still be valid where standardized techniques were employed.

Kramer (1932) found that the movement of water into hollow pawpaw petioles is considerably greater along a gradient of hydrostatic pressure than along one of osmotic pressure. This result indicates that mass flow of water (presumably through the cell walls) took place far more readily than osmosis and diffusion through the protoplasts. Similar results were obtained by Mees and Weatherley (1957*a*, 1957*b*), who concluded that appreciable mass flow of water across the cortex of tomato roots can be induced by hydrostatic pressure. Following an investigation of the effects of an increased suction tension on the passage of water through roots of *Vicia faba*, Hylmö (1958) concluded that the movement of water obeyed the Hagen-Poiseuille law for mass flow. Hylmö further deduced from deviations from this law (due to the occurrence of the Erbe phenomenon) that the width of the pores involved in the mass flow at tensions greater than 1.8 atm was of the same order as the width of the interfibrillar spaces in the cell wall as determined by X-ray, gas flow, and electrical conductance techniques, as well as by measurements made using the electron-microscope.

In regard to water movement in the intact plant, Strugger (1949) demonstrated that certain dyes which are not accumulated by the cells are swiftly transmitted along the walls of the cells of rapidly transpiring plants, and claimed this as evidence of an extrafascicular pathway of water movement.

The quantity of water which may pass through transpiring leaves may be so large that the transmission of most of the water through the protoplasts appears improbable. In species of *Eucalyptus*, for example, average rates of 50–120% of the fresh weight of the leaf per hour have been observed by Blagoveshchensky and Bogracheva (1955) in Russia at midday during summer.

(c) *Buffering Capacity of the Cell Wall*

The presence of considerable quantities of water in the cell walls of the intact leaf and the relatively high resistance of the protoplast to water movement raise the possibility that the hydrated cell wall serves as a buffer against loss of water from the protoplast during temporary adverse conditions. The drying wall would absorb water from wherever water was most readily available, i.e. from the xylem, via the walls of the intervening cells, rather than from the protoplast. During short

periods of dryness the water content of the cell wall may decrease, while the water status of the protoplast remains unchanged. In addition, the thicker the cell wall, the greater would be the buffering effect of the wall against transient drying forces.

Under steady-state conditions equilibrium would be reached between the water potentials of the cell wall, cytoplasm, and vacuole. However, methods developed recently for rapidly recording climatic factors have shown that, in nature, these factors are constantly changing, i.e. periods of steady state are rare (e.g. Swinbank 1958).

The relative contribution of the various tissues in *E. globulus* leaves to the total cell wall volume are given in Table 2. Together the epidermis and chlorenchyma, i.e. the surface from which evaporation of water occurs, compose two-thirds of the total cell wall. An increase in the thickness (and buffering capacity) of these walls would be reflected in an increase in the dry weight of the tissue. The greater dry weight/fresh weight ratios which are characteristic of plants of drier habitats, according to Pettersson and Gray (1958), may reflect only a greater amount of cell wall material in the leaves of species in drier habitats.

The results of several workers suggest that the buffering capacity of the wall water may be a factor in "hardening off" of plants when exposed to dry conditions. This may occur through an increased production of hemicellulose and pectic substances, coupled with a decrease in protein synthesis (see Clements 1937; Nezgovorova 1957; Prusakova 1960).

In the last decade, the ratio relative turgidity, i.e. the water content of a tissue/the water content at full turgor (Weatherley 1950), has found increasing use as an index of the balance between gain and loss of water by leaves. Both the name and the definition imply that only the water content of the protoplast is involved. This implication is unfortunate, since the water content of the wall must represent a significant proportion of the water content at full turgor. Indeed it seems probable that the recorded fluctuations in relative turgidity (82–92% in *Coffea arabica* (Dias and Contreiras 1958); 80–95% in potato plants (Werner 1954)) represent mainly variations in the water content of the cell walls. Slatyer (1955) records similar fluctuations in relative turgidity in cotton, peanuts, and grain soybean while the soil was moist. However, as the soil dried values of relative turgidity as low as 60% were obtained. In the latter case, the water content of the protoplast would probably be affected.

V. ACKNOWLEDGMENTS

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IONIC RELATIONS OF CELLS OF *CHARA AUSTRALIS*

V. THE ACTION POTENTIAL

By A. B. HOPE*

[Manuscript received February 13, 1961]

Summary

The effect of various ions on the resting and action potential differences in single cells of the alga *Chara australis* was studied. When the concentration of calcium ions in the external medium was changed by replacement with magnesium ions, keeping the total concentration constant, the peak level of the action potential changed reversibly. The change was about +29 mV per tenfold increase in calcium concentration, over a restricted range of the latter.

Changes in concentration of other ions did not change the peak level of action potential except insofar as the concentration of calcium in the immediate neighbourhood of the plasmalemma was modified by ion exchange.

It is suggested that the plasmalemma becomes specifically permeable to calcium during the action potential so that the peak level of potential reached is related to the ion equilibrium potential for the calcium ion. This is determined by the ratio of calcium ion activity in the cell cytoplasm to that external to the plasmalemma. Earlier published results, suggesting the chloride ion as determining the peak of the action potential, are explained in terms of the present scheme involving calcium.

I. INTRODUCTION

In previous papers (Walker 1955, 1960; Hope and Walker 1961) the resting potential difference and resistance, measured between the vacuole and external medium, have been attributed to a thin membrane (the plasmalemma) bounding the outside of the cell cytoplasm. The main factors setting the magnitude and sign of the resting potential were found to be the concentration of potassium ions and, to a lesser extent, sodium ions in the medium and the permeability of the plasmalemma to these ions. The same parameters were found to describe reasonably accurately the resistance of the cell and its change with external concentration and with current density, assuming in the analysis a linear potential gradient across the plasmalemma (Goldman 1943).

The action potential, a reversible depolarization of this membrane, occurs on stimulation by a pulse of current or by abrupt change in temperature, etc. The magnitude and form of the action potential has been the subject of much study (Osterhout 1955; Umrath 1956; Gaffey and Mullins 1958; and Findlay 1959).

The action potential in the Characeae is qualitatively similar to that in the nerve axon, which has been shown to be associated with quick changes in permeability of the membrane first to sodium and then to potassium (Hodgkin and Huxley 1952). It is of great interest to establish whether similar changes occur in cells of the

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Characeae during activity. Gaffey and Mullins (1958) reported that extra fluxes of potassium and chloride accompanied the action potential in *Chara globularis* (a cor-ticated species) and that the potential difference (p.d.) reached at the peak of the action potential was influenced by the concentration of chloride in the external medium. They therefore suggested that the permeability of the cells to chloride increased during the action potential so that the potential then approached the equilibrium potential for the chloride ion.

The present investigation is concerned with the effects of change in concentration of various ions in the medium on the action potential in *C. australis* and an alternative explanation of the results of Gaffey and Mullins is offered.

II. MATERIAL AND METHOD

Single cells of *C. australis* R.Br. var. *nobilis* A.Br. were cut from a growing culture and used either shortly after cutting or within 24 hr. Shorter and more transparent cells were chosen to enable the position of inserted micro-salt-bridges to be seen under the microscope. Cell length was usually 1–3 cm and diameter 1 mm. Internodal or, occasionally, whorl cells were used.

The experimental arrangement was essentially that used by Walker (1957) and Findlay (1959, fig. 1) where two fine glass probes filled with 0.3N KCl were inserted into the vacuole, one to record potential relative to a salt-bridge in the external medium and the other, in this case, to pass a current pulse to depolarize the resting potential until the action potential occurred. The pulse was usually of such a magnitude as to cause about 30–100 mV change in potential and of 0.3 sec duration. The time courses of the resting potential and of the action potential were recorded on a chart recorder with a full-scale response of less than 0.3 sec. Potential difference could be read from the chart to within ± 1 mV. The cells were uniformly illuminated and in a bath of flowing solution at a temperature which was constant to within $\pm 1^\circ\text{C}$ in a given experiment, and was in the range 17–21°C.

In order to test the effect of variations in concentration of a given ion it was necessary to keep the p.d. between the cell wall and medium constant, since it has been found (Hope and Walker 1961) that following an external change of total concentration, the wall potential changes quickly and the protoplast surface comes to equilibrium more slowly (half-time several minutes). The quick changes in wall potential can be eliminated by keeping the total concentration constant and increasing the concentration of one ion while decreasing that of another. The standard artificial pond water (A.P.W.1) mentioned below contained 0.1 mN KCl, 1.0 mN NaCl, and 0.5 mN CaCl_2 .

III. RESULTS

(a) Effect of Calcium Concentration

Figure 1 shows the general form of the action potential in A.P.W.1, the form being qualitatively similar to that found by other investigators. E_{oi} is the potential difference between inside and outside, with the sign of i relative to o . The level of the resting potential and the peak of the action potential are steady over a long time

interval, if stimulation is at intervals of not less than 8–10 min. Figure 2 shows the resting potential and peak of the action potential in solutions containing 0.1 mN KCl, 1.0 mN NaCl, and 5 mN ($\text{CaCl}_2 + \text{MgCl}_2$) where the calcium concentration varied from 0.3–5 mN (magnesium from 4.7–0 mN). Changes were made in the direction of increasing $[\text{Ca}^{++}]_o$ followed by a return in steps to the lowest concentration. The figure shows that, as found consistently, the resting potential is not affected by calcium, there being a steady drift from -142 to -148 mV over the duration of the experiment. However, the peak of the action potential was made more positive by increased $[\text{Ca}^{++}]_o$ in a reversible way, the new value being reached within about

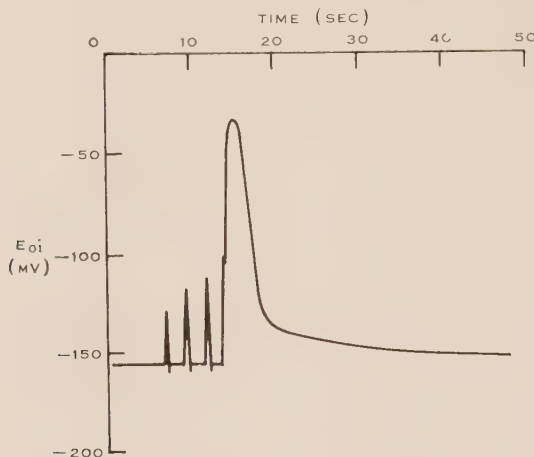


Fig. 1.—Time course of the action potential in a cell of *Chara australis*. The three spikes up to 12 sec on the time scale are the responses in p.d. to increasing pulses of current. The action potential was initiated by a fourth pulse at 14 sec which caused about 55 mV depolarization.

10 min of changing the solution. Since the points follow quite closely a line with a slope of $+29 \text{ mV}/(10 \times [\text{Ca}^{++}]_o)$, it is concluded that the cell acts as a calcium electrode at the peak of the action potential. This behaviour is found when the total (calcium+magnesium) concentration does not exceed about 6 mN and when the calcium/magnesium ratio is not less than about 1/15. Beyond those limits, with low calcium concentrations, the peak of the action potential does not remain constant but tends to become more negative and the cell may become refractory (unable to be stimulated to give an action potential). When concentrations of calcium greater than 6 mN are applied the slope of the curve of action potential *v.* $\log_{10} [\text{Ca}^{++}]_o$ becomes less than 29 mV and may be zero.

(b) Effect of Chloride Concentration

(i) *Choline Chloride*.—Changes in total concentration of choline chloride caused large changes in wall p.d. followed by slow drifts. The action potential was not steady but declined. After sufficient time the cell would become refractory. The first effect

is illustrated in Figure 3 which shows the resting potential and the action potential in 1, 3, and 10 mN choline chloride. The drift with time of the peak potential is not well illustrated here. Figure 4 shows the action potential peak over longer times, and suggests that the rate of drift of the peak of the action potential is increased by increased external concentration.

(ii) *Anion Substitutions.*—To eliminate changes in wall p.d., part of the chloride in an artificial pond water was replaced by other anions such as glucuronate, bromide, and benzenesulphonate. Table 1 records the resting potential and peak value reached during the action potential in solutions of the composition shown, after sufficient

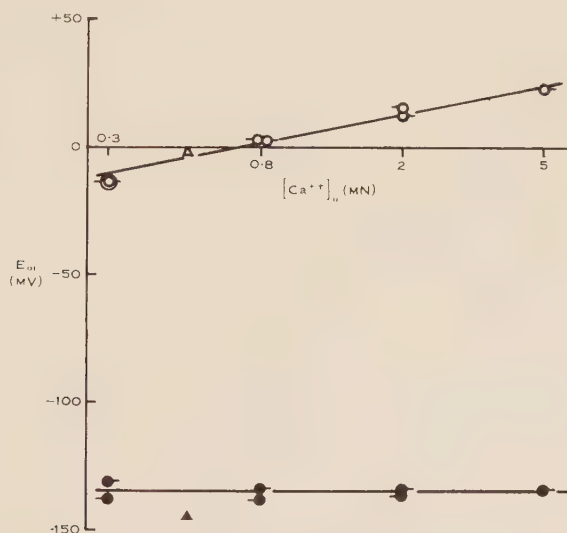


Fig. 2.—Resting potential (●) and peak level of the action potential (○), plotted against calcium concentration on a logarithmic scale. The triangles are corresponding values in A.P.W.1 (see text). Bars on symbols show direction of increasing or decreasing concentration, starting and finishing at 0.3 mN. The full line through points for action potential has a slope of 29 mV per tenfold $[Ca^{++}]_o$ change.

time (20–30 min) to reach a steady value of both, after changing a solution. Other experiments were consistent with this in that while the resting potential could be changed by anion substitutions (see also Hope and Walker 1961) the peak of the action potential was not a function of external chloride concentration.

(iii) *Changes in Chloride Concentration with Calcium Present.*—Choline chloride added to A.P.W.1 caused smaller changes in wall p.d. than those in Figure 3 and the resting potential drifted back to a value close to that in A.P.W.1. After about 20 min both resting potential and action potential kept constant values, in contrast to the results shown in Figure 4 where calcium (and some sodium and potassium chloride) were absent. The peak level of the action potential was again not a function of external chloride concentration. Such an experiment is illustrated in Figure 5.

(c) *Effect of other Cations*

(i) *Bivalent Cations*.—When calcium was completely replaced by magnesium the peak level of the action potential became more negative with time, at a rate

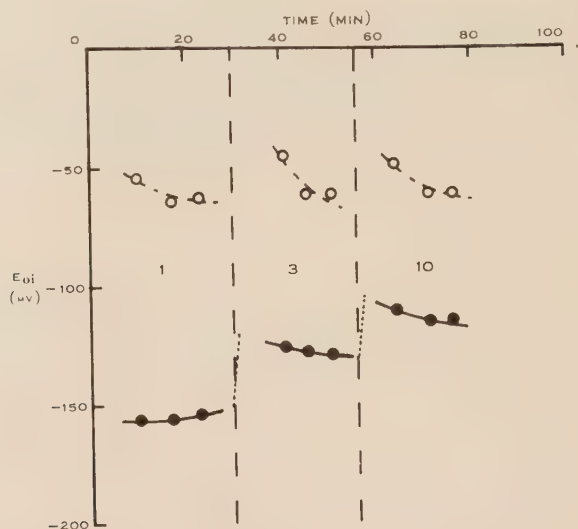


Fig. 3.—Resting (●) and action (○) potentials in solutions of choline chloride of the concentrations (mN) shown, plotted against time. Concentration was changed at times indicated by vertical broken lines.

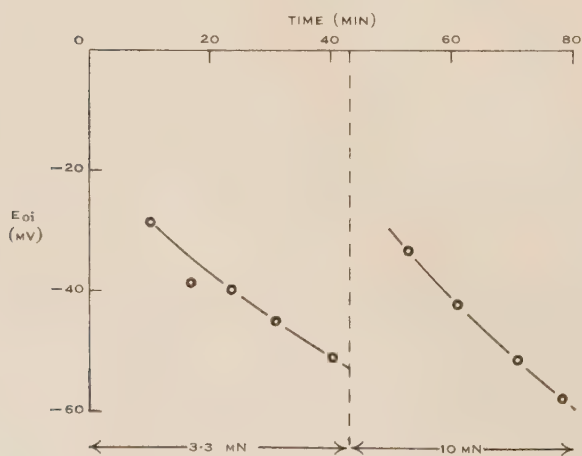


Fig. 4.—Peak value of the action potential of a cell in 3.3 mN and 10 mN choline chloride, plotted against time.

greater than with choline chloride of the same concentration. This is shown in Figure 6. The cell became refractory (*R* in the figure) to depolarizations as great

as 100 mV. The action potential was restored after replacing the calcium, either in the form of A.P.W.1 or CaCl_2 alone.

In *C. australis*, partial replacement of calcium in A.P.W.1 with barium or strontium gave a change in the action potential peak as expected on the basis of calcium electrode behaviour*.

(ii) *Monovalent Cations*.—Neither sodium nor potassium affected the peak level of the action potential in experiments where total concentration was kept constant and the metal cation replaced by choline chloride, in the presence of 0.5 mN CaCl_2 . The resting potential was little affected by sodium concentration in the range 0.3–3.0 mN, the form of the graph of p.d. *v.* $[\text{Na}^+]_o$ resembling Figure 5. Potassium affected the resting potential but the change with concentration in the presence of calcium was less than in its absence.

TABLE 1

EFFECT OF ANION SUBSTITUTIONS ON RESTING AND ACTION POTENTIALS IN CELLS OF CHARA AUSTRALIS

Time sequence from left to right. All solutions contained, in addition, sodium (4 mN), potassium (0.1 mN), and calcium (0.5 mN)

Anion	Cl^-	Cl^- Glucuronate	Cl^-	Cl^- Br^-	Cl^-	Cl^- $\text{C}_6\text{H}_5\text{SO}_3^-$	Cl^-
Concn. (mN)	4.6	0.6 4.0	4.6	0.6 4.0	4.6	0.6 4.0	4.6
Resting potential (mV)	−149	−185	−147	−149	−142	−184	−149
Action potential peak (mV)	+15	+5	+11	+5	+14	+12	+16

IV. DISCUSSION

(a) *Effect of Calcium*

Of all the ions tested only calcium was found to produce consistent changes in the peak level of the action potential in *C. australis*. The changes were of such a sign and magnitude as to suggest that, under the conditions of experiment, the cells became specifically permeable to calcium about 1–2 sec after stimulus, returning to their normal resting permeability during the next 30 sec. At the peak of the action potential the p.d. between inside and out obeyed an equation of the type:

$$E_{oi} = \text{constant} + 29 \log_{10} [\text{Ca}^{++}]_o, \quad (1)$$

which is that for a calcium electrode at 19°C.

* Specific effects of bivalent cations on the shape of the action potential, i.e. duration and rate of change of p.d., have been found here and also in *Nitella* by Findlay (personal communication).

If it is assumed that the measured p.d. contains no additive component due to the cell wall and that the cytoplasmic ion activity remains constant, equation (1) can be rewritten:

$$E_{oi} = 29 \log_{10} \{[Ca^{++}]_o/[Ca^{++}]_i\}. \quad (2)$$

$[Ca^{++}]_i$ might then be calculated from the results. In the present experiments the peak of the action potential was zero when $[Ca^{++}]_o$ was on the average 1.5 ± 0.3 mN

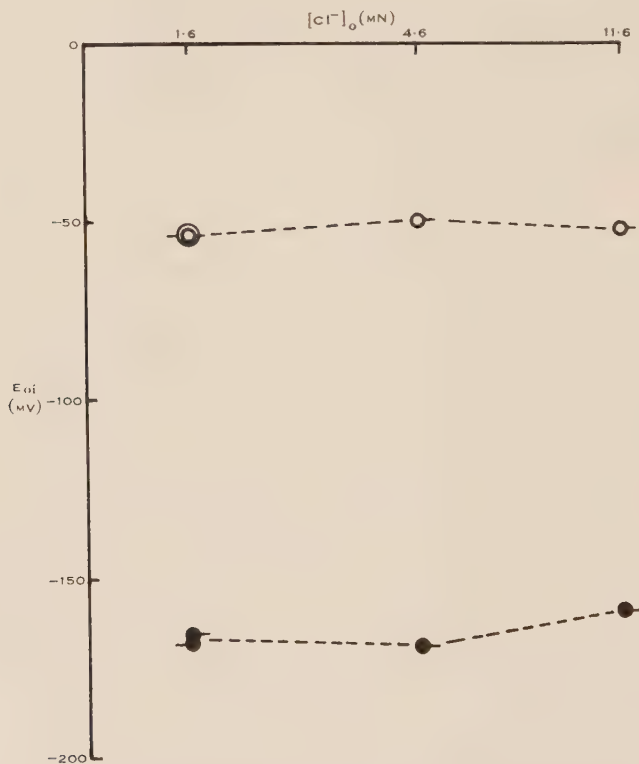


Fig. 5.—Resting potential (●) and peak of the action potential (○) of a cell in a medium in which chloride was varied by adding choline chloride to A.P.W.1 (containing 0.5 mN calcium).

in four experiments similar to that of Figure 2 (omitting one in which $[Ca^{++}]_o$ was greater than 20 mN). According to equation (2) this value can be identified with the internal (presumably cytoplasmic) calcium ion activity.

While it is not strictly permissible to compare this with measured vacuolar concentrations of calcium, it is worth mentioning that the latter was found to be 2.6 mN (average of 10 sap samples pooled) by Hope and Walker (1960).

Since the effect of calcium was obtained in the presence of concomitant changes in magnesium (or barium or strontium) concentration, the supposed increased permeability during the action potential is a very specific one and not merely an effect of bivalent cations. None of these ions had a large effect on the resting potential.

(b) *Effect of Chloride*

Increasing concentrations of choline chloride give swift changes in wall potential and also semipermanent depolarization of the resting potential (the p.d. some time after the wall change has occurred). The action potential peak was not affected by the concentration of chloride present (Figs. 3 and 5; Table 1). This is in complete contrast to the results reported by Gaffey and Mullins (1958, fig. 4) who found that the resting potential was approximately constant as the choline chloride concentration was increased from 1 to 10 mN, while the peak of the action potential became more

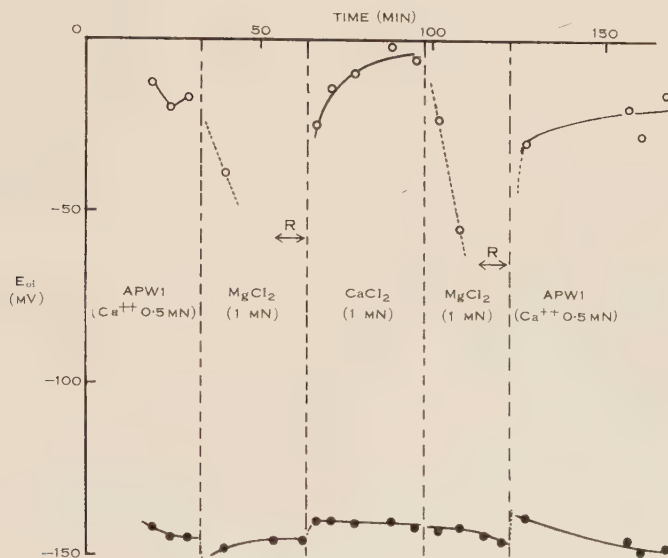


Fig. 6. Effect on the resting potential (●) and peak of the action potential (○) of substituting magnesium for calcium. At times marked *R*, the cell was refractory to large depolarizations.

negative by about 40 mV. (Its magnitude is shown as declining from 120 to 80 mV.) Such behaviour was stated as indicating that the cell became specifically permeable to the chloride ion, during the action potential.

In the present experiments, a similar decline in the action potential was found in a constant concentration of choline chloride (Fig. 4). The explanation of this, on the basis of the effect of calcium ions, is as follows. The level of the peak of the action potential is governed by the activity of calcium ions just outside the plasmalemma. On removal of a cell from a culture solution containing calcium ions into one of choline chloride (or indeed any other solution not containing calcium) the calcium ions are removed from near the plasmalemma by exchange via the cell wall Donnan phase. Exchange of bivalent counterions with monovalent ions was shown to be slow, and rates of isotopic exchange were increased by increasing the external concentration in earlier experiments with *Chara* (Dainty and Hope 1959).

The results described here are completely consistent with these considerations. The decline of the action potential reflects the decrease in calcium, not that in the

external medium, but in the immediate neighbourhood of the plasmalemma. In Figures 4 and 6, the peak potential is reduced by an exchange of calcium with choline and magnesium ions respectively. The rate of decrease of this potential is greater in 10 mM choline chloride than in 3.3 mM and, for a given equivalent concentration, is greater for magnesium than for choline. This agrees qualitatively with comparative rates of exchange in isolated wall segments of *Chara* (Dainty and Hope 1959, Table 3).

If the effective concentration of calcium is made low enough by prolonged exchange, say with magnesium (Fig. 6), the peak of the action potential would be expected to approach the level of the resting potential; stimulation while depolarizing the membrane does not initiate an action potential—the cell becomes refractory, as is observed.

When increasing concentrations of choline chloride are added in the presence of a fixed concentration of calcium, the calcium concentration next to the plasmalemma is maintained even though the wall counterions may increase in their ratio of choline to calcium. Thus the action potential peak stays almost constant with increasing choline chloride concentrations (Fig. 5).

It is thought, therefore, that the result of Gaffey and Mullins (1958) can be explained if the time sequence had been in the direction of increasing choline concentration, since the magnitude of the action potential would then appear to decrease with increasing concentration. Since, in the cells these authors used, choline did not affect the resting p.d. greatly, the net effect would be to make the peak of the action potential more negative with increasing $[Cl^-]_o$.

However, the postulated large permeability to chloride during the action potential is also indicated by the extra efflux of labelled chloride found by Gaffey and Mullins (1958) over and above the resting efflux. Extra efflux of potassium was found also. These results need to be confirmed with an uncorticated species and with "physiological" concentrations of chloride. It is entirely possible, on the other hand, that in the absence of calcium other ions may control the action potential in species other than *C. australis*.

The present experiments would predict a large current of calcium ions entering the cytoplasm during the action potential. The current flows during the action potential are being investigated by Findlay (unpublished data) under a voltage clamp, and fluxes of calcium will be measured with the aid of tracers.

(c) *Mechanism of the Action Potential*

The shape of the action potential trace has been given a detailed explanation (see Osterhout 1955) in terms of movements of potassium ions. This description is entirely theoretical and depends on the assumption that the potential change takes place at an inner "non-aqueous layer" (presumably the tonoplast) and not at the plasmalemma where it in fact occurs. Furthermore, potassium is almost in electrochemical equilibrium between the vacuole and external medium in several genera of the Characeae, which means that a sudden increased permeability to this ion would not be expected to increase the potential, as observed. The concentration of external

potassium does not affect the action potential peak but does affect the resting potential, in agreement with this view.

It is apparent from the above that the action potential can be qualitatively explained by a sudden, reversible change in the permeability of the plasmalemma to calcium. One conjecture (Mullins 1959) is that the membrane contains long narrow pores, the majority of which are filled in the resting state, in muscle or nerve, with potassium ions. The pore size is then said to change during excitation so that the majority are filled with sodium. Thus the membrane acts as a potassium electrode in one state and resembles a sodium electrode in the excited state, as observed. The ions in the pores are supposed to retain only one shell of solvation so that their sizes are related to crystallographic radii. Relative permeability of the membrane is related to relative numbers of pores filled by the various ion species, as well as ionic mobility in the pores.

This idea could be applied to *C. australis*, with calcium ions taking the place of sodium, but it is difficult to see how it could be tested, or by what molecular means the membrane effects the supposed change in pore size. Any proposed mechanism, as well as providing a reasonable explanation for the potential differences, must also account for the large increase in overall conductance (Cole and Curtis 1938) during the action potential. It is as if *more* pores (all calcium-filled) are opened and then closed, rather than as if a change in occupation of existing pores occurred.

Use of equations as simple as (1) or (2) may not be permissible if changes in permeability to other ions besides calcium are involved. Under these conditions the potential reached at the peak of the action potential would not correspond to the equilibrium potential difference for any single ion. For example, if the permeability to potassium increased sufficiently as the potential approached the equilibrium potential for the calcium ion, the observed p.d. would then tend to reach a value somewhere between the ion equilibrium potentials for these two ions, returning finally to the resting potential as the permeability to calcium declined. Experiments to test this possibility are being designed.

V. ACKNOWLEDGMENTS

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THE CULTIVATION OF ISOLATED ROOTS OF SUBTERRANEAN CLOVER AND EFFECTS OF AMINO ACIDS ON THEIR GROWTH PATTERN

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Summary

First-passage, isolated roots of subterranean clover (*Trifolium subterraneum* L.) were cultured in a basal medium of macro- and micronutrient salts and sucrose at pH 5.5. Effects of additives to this basal medium on growth rate and growth pattern of these roots and survival and growth of subcultures have been investigated.

A mixture of the vitamins thiamine, pyridoxine, and nicotinic acid doubled the rate of main axis elongation, and the addition of yeast extract further increased elongation rate. This effect of yeast extract was duplicated by casein hydrolysate and a synthetic mixture of amino acids. It was not an unspecific response to nitrogen.

High concentrations of yeast extract and casein hydrolysate inhibited main axis growth, but the latter promoted lateral growth—apparently breaking the dominance of the main axis. The effect of casein hydrolysate upon the growth of laterals could be simulated by a mixture of histidine, tryptophan, and arginine in concentrations corresponding to those in casein hydrolysate. Histidine and tryptophan strongly inhibited the growth of the main axis but they seemed to arouse growth in the lateral roots. Arginine promoted main axis growth and enhanced the growth of initiated laterals. In experiments involving growth of roots in inhibitory concentrations of a wide range of amino acids, no evidence could be found for a protective action of arginine against this inhibition.

In basal medium plus yeast extract and vitamins, the main axis meristem could be subcultured through only three transfers. Lateral roots which had grown to longer than 5 cm have been subcultured through 25 transfers in basal medium plus vitamins, yeast extract, casein hydrolysate, and glutamine. Shorter laterals and the main axis survived only one transfer in this medium.

I. INTRODUCTION

The culture of isolated roots is now a standard laboratory procedure. Over 100 species have now been grown through many transfers, many of them in simple and defined media. But many species have been reported to fail to grow, even in the best media devised (Street 1957).

Of the clovers, white and red clover have been successfully subcultured many times in simple media, without diminution in growth rate, but clones have been established only with difficulty (Bonner 1940; Dawson and Street 1959).

No previous reports of attempts to cultivate isolated roots of subterranean clover are known. Although the present attempt was not completely successful, it has at the same time contributed information on factors involved in the pattern of growth of roots and the nutritional requirements of isolated roots.

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II. MATERIALS AND METHODS

Seeds of *Trifolium subterraneum* L. cv. Bacchus Marsh, were surface-sterilized by immersion in ethanol, followed by treatment for 10 min with 0.1% mercuric chloride, and by washing alternately with several changes of alcohol and 2% saturated bromine water.

The seeds were spread in sterile petri plates containing basal medium plus 0.7% agar, held at 4°C for 48 hr to break dormancy and give uniform germination, and then incubated at 20°C. When the radicles were 3 cm long, terminal tips 5 or 10 mm long were excised from selected radicles and transferred aseptically to 40 ml of sterile medium in 150-ml conical flasks. The root cultures, one per flask, were incubated in darkness at 25°C. Although 20°C was optimal for germination, subsequent root growth proceeded fastest at 25°C; and this temperature was also optimal for the initiation of growth in subcultured roots.

Basal medium contained Bonner's macronutrient salts (Bonner 1940), White's micronutrient salts (White 1943), with the addition of 0.05 mg/l $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.13 mg/l $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, and ethylenediaminetetraacetic acid (EDTA) equivalent to the heavy metal content. Maximal growth was found to occur when the pH of the medium after autoclaving was 5.5–6.0. Hence the pH of the basal medium was adjusted to 5.5 with KHCO_3 before autoclaving. When amino acids were added to media the L-forms were always used.

The concentration of sucrose most favourable to growth was found to be 1.5–2%, and at 1.5%, sucrose was a better carbon source than glucose, fructose, mannose, or soluble starch at equivalent carbon concentration. Basal medium contained 1.5% sucrose.

Due to difficulties in producing clones and in serially subculturing the apical meristems, which will be discussed later, all experiments were based on first-passage roots derived from a single population of seed which was stored at 4°C.

The lengths of the main axes were measured *in situ* at 3-day intervals, and, where qualitative differences in growth patterns occurred, shadowgraphs were made and dry weights were taken at the conclusion of an experiment and at each subculture. Ten or 20 replications of each treatment were made.

In some experiments the growth of isolated roots was compared with that of roots of intact seedlings. The latter were obtained in sterile culture by supporting sterile seed, 1 cm above the surface of media in conical flasks, on glass slides covered with filter paper.

III. EXPERIMENTAL AND RESULTS

(a) *Influence on Growth of Supplements of Vitamins and Yeast Extract*

A mixture of the three vitamins thiamine, nicotinic acid, and pyridoxine, one or more of which are commonly required by isolated roots (Street 1957), doubled the length of isolated subterranean clover roots compared with controls in basal medium alone (Fig. 1). However, this increased rate of growth was considerably slower than that produced by the primary root tip of an intact seedling (see Fig. 5).

The addition of yeast extract (Difco, extracted with ether at pH 3) gave greater main axis length growth than the three vitamins (Fig. 1), and its effect was additive to that of the vitamins. Figure 2 shows that higher concentrations of yeast extract were inhibitory, and that the optimal concentration for the promotion of the main axis was 100 mg/l.

The experiment recorded in Table 1 demonstrates the effect of the vitamins and yeast extract upon the growth of roots through three serial subcultures of the primary root tip. Subcultures were made at 14, 24, and 34 days and the experiment concluded 45 days from first transfer. During this time the root becomes

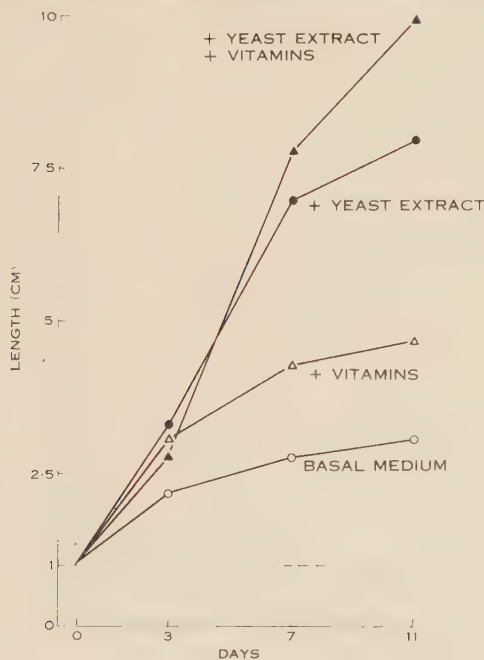


Fig. 1

Fig. 1.—Additive promotion of main axis length of isolated roots of *T. subterraneum* by yeast extract (50 mg/l) and a mixture of the vitamins thiamine (0.1 mg/l), nicotinic acid (0.5 mg/l), and pyridoxine (0.1 mg/l). Least significant differences between mean lengths:

Level	3 Days	7 Days	11 Days
5%	0.80	1.83	2.09
1%	1.08	2.46	2.80

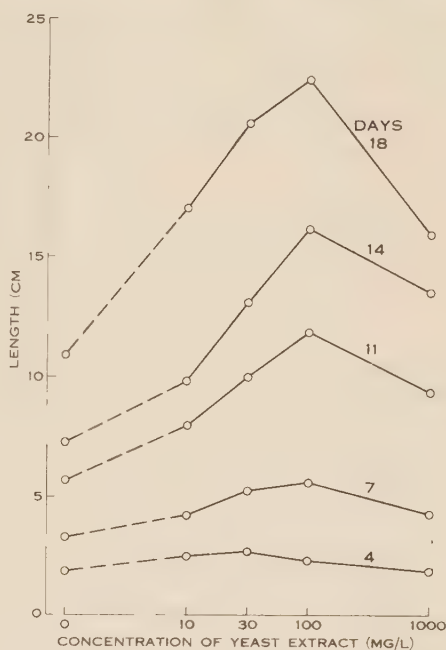


Fig. 2

Fig. 2.—Effect of yeast extract on the main axis length of isolated roots of *T. subterraneum*. Least significant differences between concentrations of yeast extract at 18 days are 2.74 at 5% level and 3.67 at 1% level.

more dependent upon externally applied substances. Growth measurements were based on the dry weights of the roots minus the weights of the 10-mm tips that had been cultured. In all treatments the growth rate diminished. Yeast extract alone produced a high initial rate, but no roots survived a second transfer. No group without nicotinic acid survived a second transfer while those with nicotinic acid

survived at least the fourth transfer. Thiamine and pyridoxine both produced a growth response, but their presence was not as essential as nicotinic acid. Figure 3 shows the average, daily, main axis length increments for the fastest (yeast extract, thiamine, nicotinic acid, and pyridoxine) and the slowest (control) groups of roots. The growth rate of the fastest group, during the first passage, is higher than any found in the literature for any species and is at least as great as that of a root attached to the plant.

In all subsequent experiments, basal medium was supplemented with 0.1 mg/l thiamine, 0.5 mg/l nicotinic acid, and 0.1 mg/l pyridoxine.

TABLE 1

EFFECT OF VITAMINS AND YEAST EXTRACT UPON THE GROWTH OF ISOLATED SUBTERRANEAN CLOVER ROOTS

Concentration of thiamine 0.1 mg/l, of nicotinic acid 0.5 mg/l, of pyridoxine 0.1 mg/l, of ether-extracted Difco yeast extract 50 mg/l

Addendum to Basal Medium	Dry Weight Increases (μ g/root/day) (average of 10 replicates)			
	1st Passage	2nd Passage	3rd Passage	4th Passage
Control	70	8	—	—
Thiamine	180	10	—	—
Nicotinic acid	180	72	54	54
Pyridoxine	180	13	—	—
Nicotinic acid + thiamine	250	141	100	80
Thiamine + pyridoxine	280	11	—	—
Nicotinic acid + pyridoxine	250	87	80	51
Nicotinic acid + thiamine + pyridoxine	260	87	89	79
Yeast extract	400	24	—	—
Yeast extract + nicotinic acid + thiamine	540	642	204	134
Yeast extract + nicotinic acid + thiamine + pyridoxine	600	536	167	66

(b) *Influence upon Growth of Supplements of Casein Hydrolysate or Amino Acids*

Yeast extract contains 15% amino acids. An attempt was therefore made to test whether it could be replaced as a root growth supplement by other amino acid mixtures.

Casein hydrolysate (Difco tryptic digest of casein) promoted main axis length growth. Figure 4 shows that, in concentrations up to 100 mg/l of casein hydrolysate, the extension of the main axis may be more than doubled. Beyond this concentration, casein hydrolysate is strongly inhibitory. A synthetic mixture of amino acids in the same proportions as in casein hydrolysate gave an identical result, which ruled out the possibility of the effects of casein hydrolysate being due to peptides or to impurities in the casein or the trypsin.

Even low concentrations of many individual amino acids are notoriously toxic to plant organs (Street 1957), so it seemed fortuitous that the balance of amino acids in casein hydrolysate permitted a growth-promoting effect to manifest itself.

Casein hydrolysate and yeast extract together did not promote main axis growth above that obtained with casein hydrolysate or yeast extract alone (Fig. 5). The same initial growth rate was also obtained for the root main axis of intact seedlings, grown in basal medium plus vitamins.

The particular amino acids in casein hydrolysate which are responsible for stimulating the growth of the root main axis have not been sought exhaustively, but it has been found that arginine and possibly glutamine and asparagine are involved. This is not an unspecific nitrogen response, since the basal medium contains

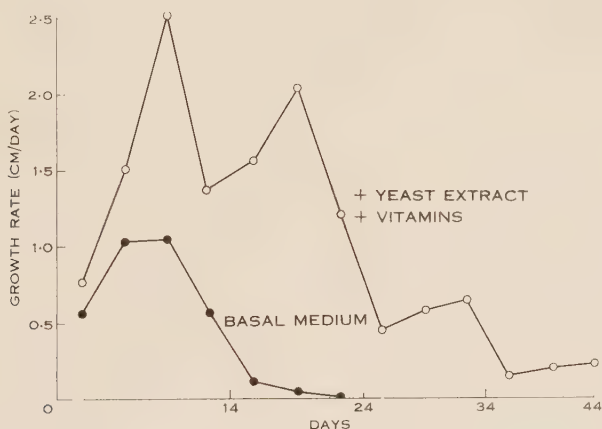


Fig. 3.—Effect of yeast extract plus vitamins on the daily length increment of isolated roots of *T. subterraneum* through three serial subcultures. Subcultures were made 14, 24, and 34 days from first transfer. Concentrations of vitamins are: thiamine, 0.1 mg/l, pyridoxine, 0.1 mg/l, and nicotinic acid, 0.5 mg/l.

$2.8 \times 10^{-3}M$ nitrate. In Figure 4, growth after 13 days is doubled by casein hydrolysate at 30 mg/l, which is equivalent to $0.3 \times 10^{-3}M$ nitrate. Doubling the concentration of nitrogen in the basal medium by adding nitrate, ammonium, urea, or glutamic acid gave no increase in growth.

(c) Influence of Casein Hydrolysate and some Amino Acids upon Growth Pattern

Casein hydrolysate, in addition to its effect on the length of the main axis, has a striking qualitative effect on the growth pattern of the root. Subterranean clover roots, both in the isolated state and attached to the seedling, strongly exhibit main-tip dominance. No lateral roots emerge within 5–8 cm of the main axis tip, and no laterals within 20–30 cm of the tip grow at a rate comparable with that of the main axis tip. As they become older, laterals seem to undergo a discrete change which permits them to grow more rapidly. In the isolated root, lateral roots are initially

much thinner and grow at only 5–10% of the rate of the main axis, and such lateral meristems when excised, nearly always fail to grow.

As the laterals pass into the phase of rapid growth, they thicken and the zones of cell division and elongation become much longer. Casein hydrolysate has a striking effect in promoting the growth of the laterals (Fig. 6; Fig. 7(i)). Figure 6

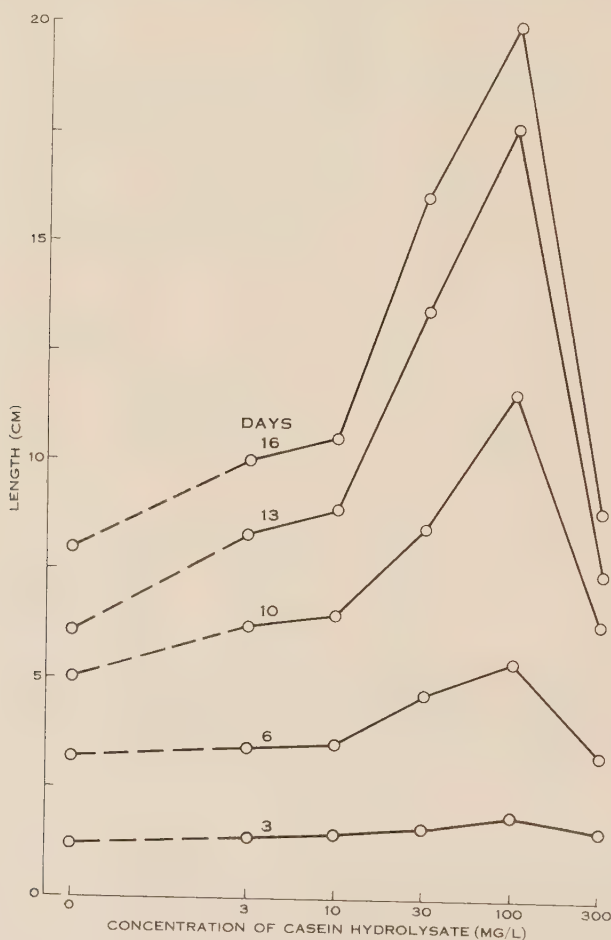


Fig. 4.—Effect of casein hydrolysate (tryptic digest of casein) upon main axis length of isolated roots of *T. subterraneum*. Least significant differences between concentrations of casein hydrolysate at 16 days are 2.72 at 5% level and 3.64 at 1% level.

shows the stimulation of the number of laterals at low casein hydrolysate concentrations. At higher concentrations, supra-optimal for main axis length and lateral number, the total dry weight continues to increase, i.e. the growth of laterals already present is preferentially promoted. Table 2 shows that at 140 mg/l, casein hydrolysate inhibited the growth of the main axis and while the number of emerged laterals is not different, the average total length of laterals is increased fourfold.

A synthetic mixture of amino acids equivalent to casein hydrolysate had an identical effect on the growth of laterals. Identification of the amino acid or combination of amino acids responsible, would involve testing 24 amino acids singly, two at a time, three at a time, etc. and would be a tremendous task. This was not undertaken. Instead, blocks of amino acids were tested in combination, ignoring interactions, and where promoting effects on lateral growth were indicated, the blocks were broken down into individual amino acids and simple combinations of them.

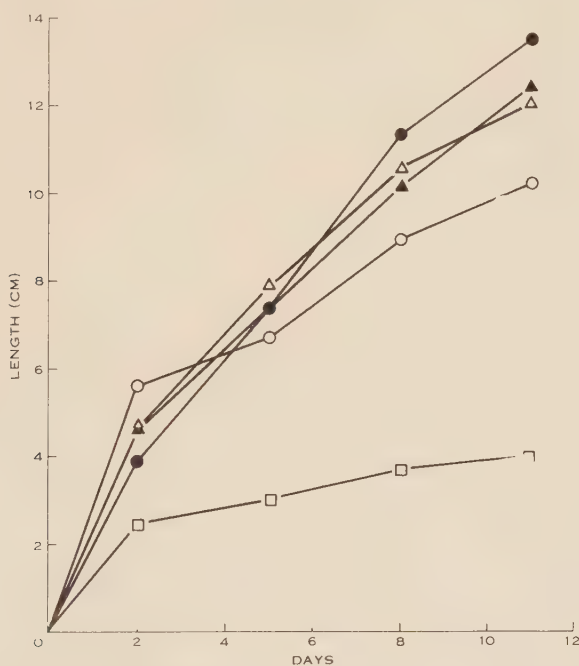


Fig. 5.—Comparison of the effect of yeast extract and casein hydrolysate, alone and together, on the main axis length of isolated roots of *T. subterraneum*, with the growth of roots of intact seedlings. □ Isolated roots in basal medium plus vitamins. ● Isolated roots in basal medium plus vitamins plus yeast extract (50 mg/l). ▲ Isolated roots in basal medium plus vitamins plus casein hydrolysate (30 mg/l). △ Isolated roots in basal medium plus vitamins plus yeast extract (50 mg/l) and casein hydrolysate (30 mg/l). ○ Intact seedlings in basal medium plus vitamins. Least significant differences between treatments:

Level	2 Days	11 Days
5%	1.37	3.49
1%	1.83	4.69

Although other possibilities exist, it was found that the effect of casein hydrolysate on laterals could be simulated by a mixture of histidine, tryptophan, and arginine in concentrations corresponding to their concentrations in casein hydrolysate (see Table 3 and Fig. 7). Histidine and tryptophan are both strongly inhibitory to the growth of the main axis, but they seem to initiate rapid growth in the laterals. Added arginine not only partially overcomes the suppression of growth of the main apex by tryptophan and histidine, but it also enhances growth in the initiated laterals

(cf. Fig. 7). Figure 8 shows that arginine can promote the growth of the main axis inhibited by histidine plus tryptophan and can increase the dry weight of roots under these conditions.

Goldacre (1957) found that 2,6-diaminopurine, at concentrations of $3 \times 10^{-9}M$, and below, promoted the growth of the main axis of *isolated* subterranean clover roots without any change in growth pattern. In *whole* seedlings the growth of the root main

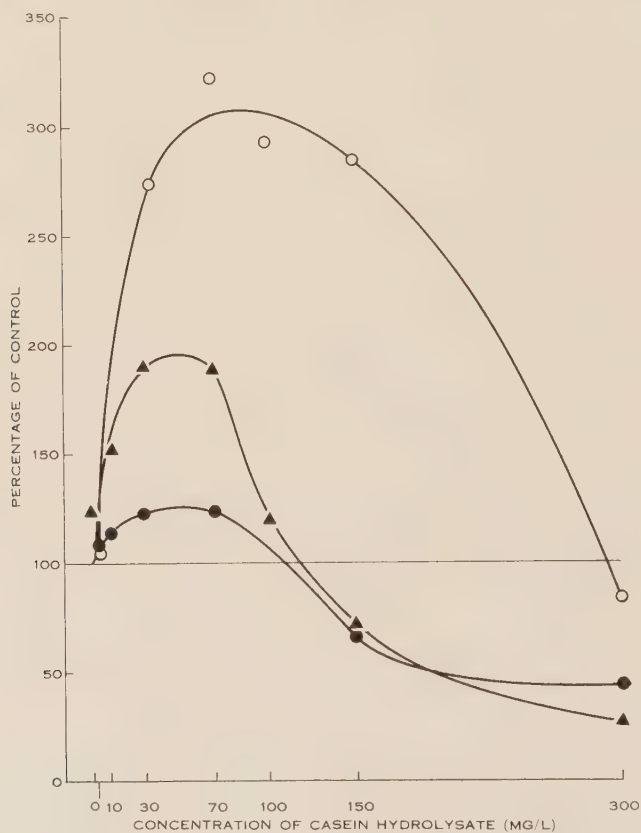


Fig. 6.—Effect of casein hydrolysate upon dry weight (○), number of lateral roots (▲), and main axis length (●) of isolated roots of *T. subterraneum* grown for 11 days in basal medium plus vitamins.

axis was stimulated by low concentrations while the laterals were stimulated by concentrations supra-optimal for main axis growth. As this differential promotion occurs only in the intact seedling it was suggested that substances normally produced in the aerial portions of the plant may be necessary for the stimulation of secondary root meristems by 2,6-diaminopurine. Because of their effect on lateral root development discussed above it seemed possible that these substances may in fact be tryptophan, histidine, and arginine. Therefore first-transfer roots were grown in medium supplemented with 16.8 mg/l tryptophan, 2.98 mg/l histidine, and 6.24 mg/l

arginine, with and without $3 \times 10^{-9}\text{M}$ or 10^{-7}M 2,6-diaminopurine. The amino acids at these concentrations stimulate lateral growth, but they had no effect upon the growth of laterals in concentrations of 2,6-diaminopurine which are supra-optimal for main axis growth. That is, the amino acids did not substitute for the tops.

TABLE 2

EFFECT OF CASEIN HYDROLYSATE ON THE GROWTH OF THE MAIN AXIS AND THE GROWTH OF LATERAL ROOTS OF SUBTERRANEAN CLOVER

Incubated 13 days at 25°C

Treatment	Average Length of Main Axis (cm)	Average Number of Emerged Laterals	Average Total Length of Laterals (cm)
Control	15.3	30.2	7.5
Casein hydrolysate (140 mg/l)	11.0	36.0	27.7

(d) *Growth in the Presence of Individual Amino Acids and the Effects of Arginine*

It was observed that arginine promoted subterranean clover root growth, alone and in the presence of other amino acids although the effect was variable. Harris (1953) had observed the interaction of amino acids whereby one amino acid

TABLE 3

EFFECT OF AMINO ACIDS UPON THE GROWTH OF ISOLATED SUBTERRANEAN CLOVER ROOTS

Incubated 10 days at 25°C. The concentrations of amino acids are equivalent to those found in 140 mg/l casein hydrolysate

Amino Acid added to Basal Medium plus Vitamins	Dry Weight per Root (mg)		Length of Main Axis (cm)*	
	Alone	With Arginine (6.24 mg/l)	Alone	With Arginine (6.24 mg/l)
Control	4.01	5.86	22.7	24.3
Tryptophan (16.8 mg/l)	4.23	7.96	11.2	15.2
Histidine (2.98 mg/l)	2.85	4.61	8.0	14.0
Tryptophan + histidine	3.86	9.88	6.5	15.8
Synthetic casein hydrolysate (140 mg/l)	11.19	—	25.3	—

* L.S.D. between mean lengths of main axis: 4.15 at 5% level, 5.52 at 1% level.

relieved the inhibition by another amino acid of the growth of roots of *Avena* embryos. Gullino *et al.* (1955) found that the toxicity of individual amino acids to rats was reduced by arginine. The inhibition of growth of tobacco pith callus by valine has been found to be relieved by isoleucine (Sandstedt and Skoog 1960).

It was of interest then to measure the main axis elongation and dry weight increases of first transfer roots after 14 days growth in a series of concentrations ($2 \times 10^{-6}M$ – $128 \times 10^{-6}M$) of amino acids without and with $10^{-3}M$ arginine. Arginine alone consistently increased the main axis length and dry weight of roots, but the amount of this increase varied between 5 and 100% on different occasions. No inhibition of growth was obtained with arginine at concentrations up to $10^{-3}M$.

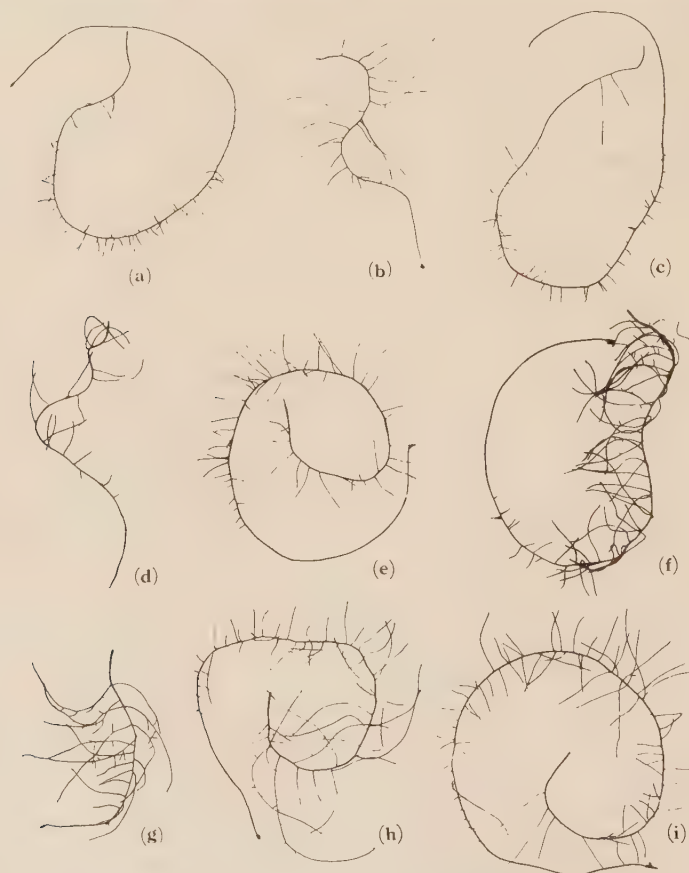


Fig. 7.—Shadowgraphs of isolated roots of *T. subterraneum* grown for 12 days in basal medium containing vitamins plus: (a) no addendum; (b) histidine, 2.98 mg/l; (c) arginine, 6.24 mg/l; (d) tryptophan, 16.8 mg/l; (e) histidine, 2.98 mg/l, plus arginine, 6.24 mg/l; (f) tryptophan, 16.8 mg/l, plus arginine, 6.24 mg/l; (g) histidine, 2.98 mg/l, plus tryptophan, 16.8 mg/l; (h) histidine, 2.98 mg/l, plus tryptophan, 16.8 mg/l, plus arginine, 6.24 mg/l; (i) casein hydrolysate, 140 mg/l. The concentrations of amino acids are equivalent to those found in casein hydrolysate at a concentration of 140 mg/l.

Those amino acids which did not influence growth were: glutamic acid, glutamine, glycine, and asparagine. Phenylalanine, isoleucine, hydroxyproline, tryptophan, aspartic acid, and serine inhibited at all concentrations or were ineffective at low concentrations and inhibited at high concentrations. Lysine, methionine, leucine, histidine, threonine, alanine, cysteine, valine, tyrosine, and proline slightly promoted

growth at a low concentration and inhibited at high concentrations. Arginine had the same general effect in all experiments. The promotion of growth obtained in the presence of arginine alone was more or less maintained in all concentrations of another amino acid. There was no evidence for synergism or of a protective effect of arginine from the inhibition by other amino acids.

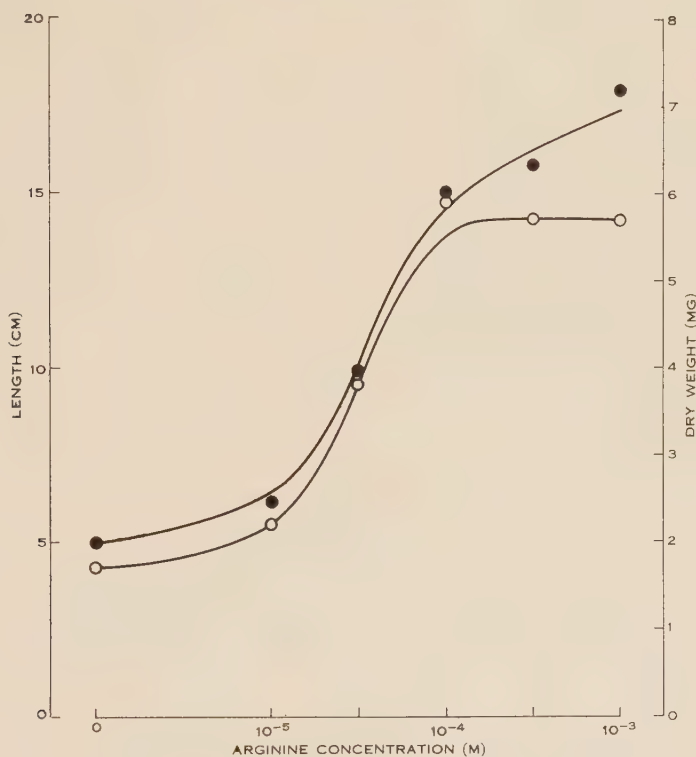


Fig. 8.—Effect of arginine upon the main axis length (●) and dry weight (○) of isolated roots of *T. subterraneum* cultured for 14 days in basal medium plus vitamins, histidine (2.98 mg/l), and tryptophan (16.8 mg/l). In absence of amino acids, weight was 0.5 g and length was 12.5 cm. Least significant differences between arginine concentrations:

Level	Length	Dry Weight
5%	1.57	0.74
1%	2.09	0.99

(e) Subculturing of Subterranean Clover Roots

As was seen in Figure 3, the addition of yeast extract plus the three vitamins thiamine, pyridoxine, and nicotinic acid did not sustain the main axis meristem beyond three transfers. The following attempts were made to increase the number of viable transfers.

Street (1954) had shown that the substance α -(1-naphthylmethylsulphide)-propionic acid (NMSP) overcomes the "aging" of the main axis meristem of tomato

roots. Although NMSP increased the growth of the main axis of first-transfer subterranean clover roots, it did not improve the growth of subsequent subcultures. The possibility that substances in seeds, contributing to root growth, would diffuse out of seeds and influence the growth of excised roots, was investigated. Diffusates of subterranean clover seeds were found to be inhibitory to isolated roots. Other substances which did not increase the growth of subcultured main axis meristems were pantothenic acid, riboflavin, biotin, inositol, folic acid, and *p*-aminobenzoic acid.

Street and Roberts (1952) found that the main axis meristem of tomato could be subcultured a limited number of times and obtained clones by excising laterals alternately with the main axis meristem. This procedure was not effective with subterranean clover because the growth of the laterals is also unreliable. As pointed out previously, the laterals of subterranean clover roots undergo an abrupt change from very slow to rapid growth. The ability of laterals in these two states to be subcultured has been studied and compared with the performance of the main axis meristem. The supplements to the basal medium plus vitamins were 50 mg/l yeast extract, 70 mg/l casein hydrolysate, and 50 mg/l glutamine. It was found that the main axis and laterals less than 5 cm long could be subcultured only once. Laterals longer than 5 cm, however, were subcultured 25 times over a period of two years, but the chance of an individual tip surviving a transfer was only 50%. Experience showed that the lateral root tips which survived were those which floated on the medium after excision.

IV. DISCUSSION

Excised root culture is still mainly in the descriptive, comparative phase of investigation. This paper adds to the descriptive facts with particular reference to a peculiarity of subterranean clover roots: the strong dominance of the main axis tip over the laterals.

Other workers have shown similar effects of exogenous amino acids upon the growth of isolated roots, particularly groundsel and red clover roots (Skinner and Street 1954; Charles and Street 1959; Dawson and Street 1959; and Harris 1959).

The results emphasize the complexity of the isolated root as a growth system. Although isolated roots are sometimes classified as "tissue cultures", they are more accurately described as organ cultures, and these experiments suggest that the isolated subterranean clover root is an organ of changing synthetic capacity. Lateral meristems, either on the intact plant or on an isolated root in the best medium available, grow slowly and make no further growth when subcultured. After a time these laterals undergo an abrupt change which produces rapid growth both on the intact plants and on an isolated root and allows a reasonable chance of successful subculture. A combination of histidine, tryptophan, and arginine hastened the transformation of lateral meristems from sluggish to active growth but no clues could be found concerning the reason for the transformation *in situ*. Experiments on subculturing the two types of lateral and the primary root suggested that the state of the meristem at the time of excision, rather than the exogenous nutritional conditions determines growth behaviour.

V. ACKNOWLEDGMENT

The statistical treatment of the data was done by Mr. G. A. McIntyre, Division of Mathematical Statistics, C.S.I.R.O.

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STUDIES ON PHYTOALEXINS

III. THE ISOLATION, ASSAY, AND GENERAL PROPERTIES OF A PHYTOALEXIN FROM *PISUM SATIVUM* L.

By I. A. M. CRUICKSHANK* and DAWN R. PERRIN*

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Summary

An antifungal compound which conforms to Müller's definition of a phytoalexin has been isolated from the endocarp tissues of detached pea pods inoculated with *Monilinia fructicola*. The same compound has been identified in inoculated organs of growing pea plants. This compound, which is an abnormal metabolite of pea tissues, has been named "pisatin".

The details of a quantitative bioassay for the measurement of fungitoxicity are described.

A chemical method for the estimation of pisatin in aqueous solutions of plant extracts is given.

Some of the physicochemical properties of pisatin are:

- (1) It has a molecular formula $C_{17}H_{14}O_6$; the crystals are colourless, m.p. 72°C .
- (2) Solutions of pisatin have a characteristic ultraviolet absorption spectrum, λ_{max} 286 m μ and 309 m μ in ethanol.
- (3) The solubility of pisatin in water at 23°C is 0.03 mg/ml.

Pisatin is stable to heat treatments including autoclaving (15 lb, 20 min). It is also stable to visible light but its antifungal activity is destroyed by high-energy radiation in the 253.7 m μ range of ultraviolet light, and by sunlight.

Pisatin is fungistatic at the concentration (mean value 67.5 $\mu\text{g/ml}$) it normally occurs in extracts (diffusates) from infected plant tissues. Although at this concentration no toxic effect could be demonstrated towards leaf and pod cells, the growth of wheat roots was significantly inhibited.

The results are discussed in relation to other compounds which appear functionally similar to pisatin in that they are abnormal metabolites with fungitoxic activity and are formed as a response to fungal infection.

I. INTRODUCTION

Hiura (1943), Gaumann, Braun, and Bazzigher (1950), Müller (1956, 1958), Uehara (1958*a*, 1958*b*, 1958*c*), and Condon and Kuć (1960) have each demonstrated the formation of abnormal metabolites, with antifungal activity, as a result of the interaction between fungi and specific tissues of certain higher plants. In each case, with the exception of that of Hiura (1943), the authors have suggested that these compounds are of direct significance to the natural disease resistance of the tissues in which they are formed. Kubota and Matsuura (1953) established the chemical structure of the compound isolated by Hiura (1943). This compound, ipomeamarone, has recently been reinvestigated by Akazawa (1960), who suggests it may also play

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a role in the natural disease resistance of the tissues in which it occurs. Gaumann's work has been followed up by Boller *et al.* (1957), who isolated one of the components of the antifungal complex, namely orcinol. Condon and Kuć (1960) in the course of their work isolated a third antifungal compound from infected roots of carrots.

We now report the isolation and general properties of an abnormal metabolite with antifungal properties, not detected in healthy tissues of *Pisum sativum* L. but produced in hypersensitivity studies similar to those described by Müller (1956, 1958). The name "pisatin" has been proposed for this new compound (Cruickshank and Perrin 1960).

II. EXPERIMENTAL AND RESULTS

(a) Sources of Antifungal Activity

(i) *Detached Pods*.—For the purpose of isolating the compound primarily responsible for the antifungal activity in one of the supplementary host-parasite combinations used by Müller (1958), and in order to obtain sufficient quantity of it for chemical identification, a large-scale inoculation of pea pods was carried out. Three hundred pounds of detached opened pods of field-grown peas (cv. Greenfeast), in 20-lb batches, were set out in large covered "Perspex" trays, and inoculated with a spore suspension (concn. 4×10^5 spores/ml) of *Monilinia fruticola* (Wint.) Honey (synonym *Sclerotinia fruticola* (Wint.) Rehm). The inoculum was applied with constant-flow syphon bottles at approximately 1 ml per pod. The inoculated pods were then incubated for 40 hr at 20°C and, after this period, the liquid ("diffusate") remaining on the inoculated areas of the endocarp tissues was drawn off under vacuum and collected. The inoculum was replaced by sterile water and left a further 24 hr. Sterile water applications were repeated up to three times, depending on the physiological condition of the pods. In each case the liquid was removed and tested for the presence and concentration of antifungal substance before being added to the diffusate bulk.

The pisatin concentration of diffusates collected after 40 hr was not a constant value. If the solutions of diffusate recovered from the 15 batches of peas used in the above extraction series are considered, the concentration range was 52–97 µg/ml (mean value 67.5 µg/ml). With the exception of one or two of the lowest values, these concentrations were sufficient to completely inhibit germination of *M. fruticola* under the conditions of the bioassay described below. The lowest values, although inadequate to prevent germination, were, however, in excess of the concentration required to prevent colony growth of this organism *in vitro* (Cruickshank, unpublished data).

(ii) *Organs Attached to Growing Plants*.—The waxy bloom was removed from leaves and stems of pea plants growing in pots by gently rubbing them between the fingers. They were then inoculated by placement of drops of spore suspension of *M. fruticola* on these areas, and incubated under conditions of high humidity (temp. 20°C, R.H. 100%, time 40 hr). Pods attached to plants, growing both in the glass-house and field, were inoculated by injection of a spore suspension of *M. fruticola* directly into the cavity of the pods with a hypodermic syringe, and left for 40 hr. Care was taken to prevent contact of the injection wound with the spore suspension.

Diffusates were collected from the leaf and stem inoculations as described for detached pods and analysed in the same way (see below). Endocarp analysis (see below) was necessary in the case of injected pods to identify and determine the concentration of the antifungal compound formed in the course of these tests. In each instance pisatin was detected at concentrations comparable to those in the diffusate solutions in the detached pod experiments.

(b) *Bioassay of Pisatin*

(i) *Antifungal Activity of Extracts*.—Supernatants of centrifuged diffusates were assayed for antifungal activity, using a technique similar to that described by Müller (1958). For the purpose of guiding the chemical extraction and purification of the antifungal component from the diffusate, and to determine whether a close correlation existed between the antifungal compound and the light-absorbing component in the extract, a more quantitative bioassay was developed. The following procedure was followed, using *M. fruticola* as the test organism.

Three spore suspensions (concn. 4×10^5 spores/ml) were prepared by blending the spores of three lots of four 5–7-day-old slant cultures grown at 20°C on potato dextrose agar in the dark. The spore suspensions were prepared in the cold in order to prevent premature germination. Six tubes of double-autoclaved Difco prune agar were dissolved and cooled to 40°C in an accurately controlled water-bath with stirrer. 1.5 ml of each spore suspension blend were used to seed each of two agar tubes. Each tube was held in the bath for 30 sec and the contents were then poured into a level flat-bottomed sterile petri dish (9 cm dia.). The latter were stored at 2°C prior to use.

Solutions to be tested were prepared either in water or 5% ethanol, using a dilution series with an 0.75 dilution interval. The dilution series, consisting of six solutions and a water control, were dispensed into open watch-glasses (0.45 ml/watch-glass) set out on trays lined with moist filter-paper, and covered with a "Perspex" sheet. The seeded agar in the petri dishes was cut into 5 by 5 by 1 mm blocks with a sterile stainless steel disk-cutter. One agar block from each of the six petri dishes was placed into each test solution (for statistical purposes each agar block was treated as a single replicate). The assays were carried out at 20°C in the dark. After 6 hr the spores were killed by addition of a drop of 1% formalin and cotton-blue lactophenol. Percentage germination was determined by classifying the first 100 spores in each agar block into germinated and non-germinated. The convention, spore tube length > spore diameter = germination, was used.

(ii) *Statistical Transformation of Bioassay Results*.—The mean percentage of germinated spores, corrected for control germination was plotted against log concentration of extract, and a free-hand curve drawn. The curve was not of the symmetrical form given by the cumulative normal distribution. It had a long arc towards maximum germination relative to the short opposing arc approaching zero germination. In consequence, transformation to probits did not give a linear relation. An empirical transformation to achieve this end was determined graphically by drawing a straight line through the point of median germination and cutting the base line at a concentration slightly in excess of that which gave no germination. Scores to correspond to the percentage germinations were read off the percentage scale as

the ordinates of the line and curve at the same concentration. The score at the median was 50.

The curvilinear relation of scores to percentages was graduated and graphed. The variance of a score corresponding to a percentage P , to a first approximation, is given by

$$P(100 - P)/n.(ds/dP)^2,$$

where (ds/dP) is the slope of the tangent to the curve at entry P and n is the number of spores on which P is based. The inverse of the variance is the weight to be given the observation in fitting a linear regression line to scores on log concentration. From the regression line obtained the ED_{50} value of the test solution was read off. The fungitoxicity of a solution in phytoalexin germination units (PAG units) is given by $(100/ED_{50})$.

(c) Analysis of Bioassay

(i) *Test of Homogeneity of Dosage Response Slope.*—Weighted regression lines were fitted for seven samples and departures of residues from expected values tested, using χ^2 . Two of the lines showed some departure from expectation, which could be due to factors other than the binomial variation in the experimental set up, or to non-linearity of the score/log dilution line. From the plot of germination score values (Fig. 1) for the several dilution series, however, no systematic departure from linearity was evident. An analysis of variance (see Table 1) made of the homogeneity of slopes in the lines and the deviation of slopes from the mean slope was not significant. Germination percentages corresponding to the score values are also given in Figure 1.

(ii) *Test of Variation.*—From the analysis of variance (Table 1), the mean value for the within-test variation was shown to be 1.78 times the variation one would expect from binomial variation. This corresponds to a mean value of 4.4 reported by McCallan, Wellman, and Willcoxon (1941), using the standard slide germination technique.

A series of reference sample values were compared in eight experiments carried out over a period of 6 weeks. The variation between PAG values obtained for the series relative to the expected variation deriving from the binomial variation within dilutions was 5.41. Using the standard slide germination technique McCallan, Wellman, and Willcoxon (loc. cit.) reported an equivalent value of 23.7 where *M. fructicola* was used as the test fungus and inorganic compounds were bioassayed.

(d) Chemical Assay of Pisatin

The characteristic ultraviolet absorption spectrum of pisatin, and the partition of pisatin between light petroleum and water have been used to develop a chemical assay for pisatin in inoculum diffusates. 5-ml aliquots of diffusates were centrifuged at 1900 g for 10 min and the supernatant liquid transferred to 6 by $\frac{3}{4}$ in. glass-stoppered tubes. The sediment in the centrifuge tube was washed with 1 ml of distilled water, centrifuged at 1900 g for a further 5 min, and the wash fluid transferred to the diffusate supernatant. The combined liquids were then extracted four times with an equal volume of light petroleum (b.p. 55–60°C) and the combined extracts

taken to dryness at a temperature less than 40°C *in vacuo*. The residue of pisatin was dissolved in 5 ml of ethanol (redistilled, suitable for spectrophotometry to $260\text{ m}\mu$),

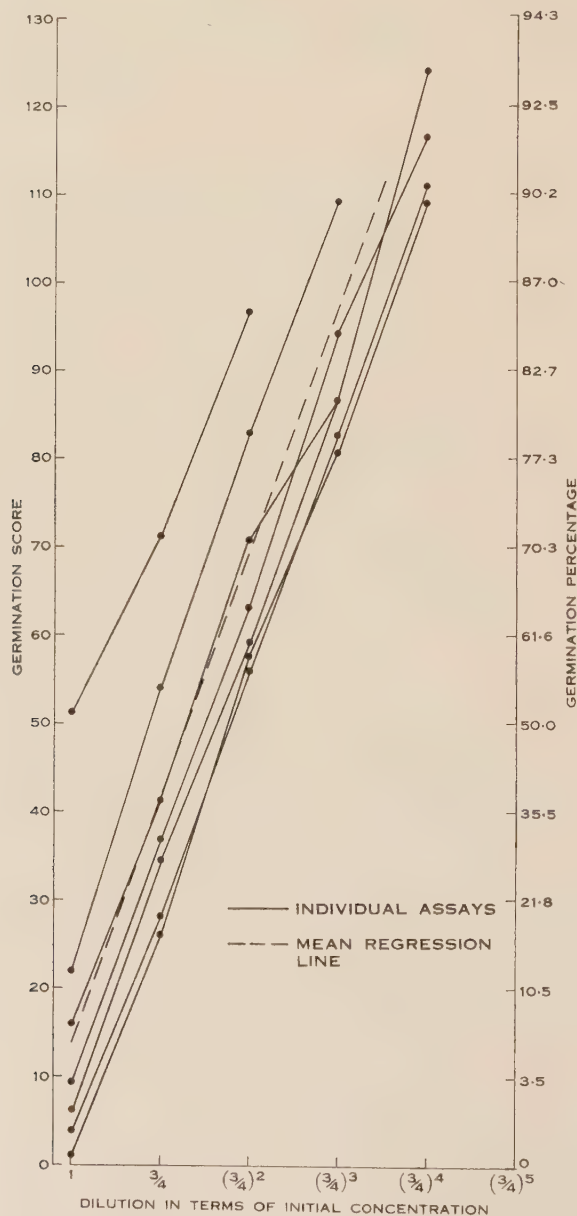


Fig. 1.—Dosage response curves of pisatin solutions against *M. fructicola* (for details of bioassay and statistical transformation see Section II(b) of text).

and the optical density (O.D.) of the resulting solution measured at $286\text{ m}\mu$ and $309\text{ m}\mu$ using a 1-cm cell in an ultraviolet spectrophotometer. For spectrophotometry,

samples were diluted as required to give O.D. readings within the accurate range of the instrument used. The concentration of pisatin in diffusates was calculated from the O.D. at 309 m μ , taking an O.D. value of 1.00 for a 5-ml solution as equivalent to 43.8 μ g/ml of the original solution. When pisatin was the only light-absorbing species present the ratio O.D._{309 m μ} to O.D._{236 m μ} was 1.47.

(e) *Biophysical Correlation*

In the early stages of this work biological activity was the only criterion available to guide the extraction of the antifungal compound in the diffusates. Once purified extracts were available, however, it became apparent that the ultraviolet absorption spectrum of the biologically active fraction could be used for its identifi-

TABLE I
ANALYSIS OF VARIANCE

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	<i>F</i>
Mean slope	1	6963.66	6963.66	} 1.95*
Variation between slopes	6	20.85	3.48	
Residues	20	35.57	1.78	

* Not significant.

cation and estimation, provided a high level of correlation existed between the biological and physical tests.

Results from four separate experiments were considered. The PAG values of the samples in each experiment (three sets in water, one set in 5% ethanol) were plotted against the O.D._{309 m μ} values in ethanol. The results are presented in Figure 2. This corresponds to a correlation coefficient between 0.8 and 0.9 and strongly suggests that the light-absorbing species present corresponds to the fungitoxic component of the solutions. This was later confirmed by the isolation and testing of pure samples of pisatin.

(f) *Isolation and Purification of Pisatin*

The diffusate collected from the endocarp of pea pods inoculated as described above was clarified by centrifuging. It was then extracted four times with an equal volume of light petroleum (b.p. 55–60°C) (Müller 1958). The aqueous residue after these extractions was assayed as described above and shown to be non-toxic. Endocarp tissue stripped from the inoculated pods was extracted by maceration of this tissue in 80% ethanol at room temperature. The ethanol extract was centrifuged and the supernatant collected. The latter was then evaporated to small volume in a rotary film evaporator and the alcohol replaced by an appropriate volume of water.

The resulting aqueous solution was extracted with light petroleum as described for diffusates.

The various constituents from light petroleum extracts from diffusates and the corresponding endocarp tissues were separated on paper chromatograms (Whatman No. 3 paper; solvent *n*-propanol-water, 20:80 v/v; ascending). The paper used was purified by chromatographic washing with acetic acid and ammonia (Isherwood and Hanes 1953). The chromatograms after development were eluted with 50% ethanol (v/v). The ethanol was then removed and the eluates tested for antifungal activity in aqueous solution. In all extracts with initial antifungal activity a fungus-inhibiting

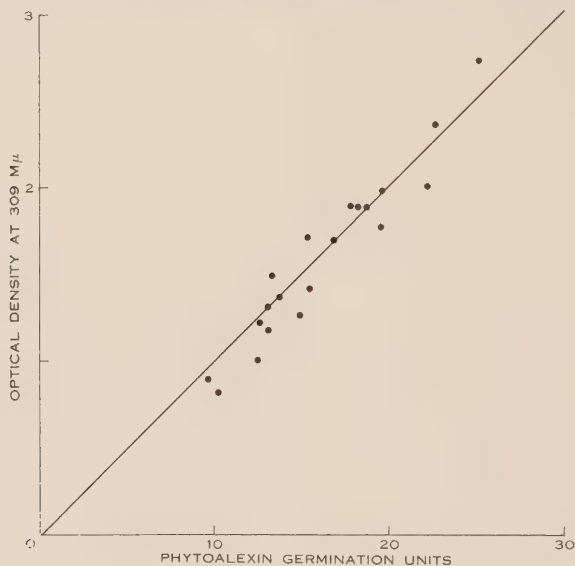


Fig. 2.—Biophysical correlation diagram.

fraction was found at R_F 0.60–0.70. A characteristic ultraviolet-light-absorbing species was also observed in this fraction. No other antifungal components could be detected. Representative tests for other groups of compounds on the chromatograms described and on chromatograms developed in other solvent systems indicated that the extract of the diffusate was essentially chromatographically homogeneous.

An antifungal compound corresponding to the above fraction was subsequently separated from a light petroleum extract by repeated recrystallization from light petroleum and from aqueous ethanol. This extraction procedure, used in the large-scale preparation of pisatin from the diffusate solution described in Section II(a)(i), yielded a colourless needlelike crystalline compound with a melting point of 72°C. The yield from 300 lb of pea pods was approximately 5 g.

(g) *Physical and Chemical Properties of Pisatin*

A preliminary account of some of the physicochemical properties has already appeared (Cruickshank and Perrin 1960). Further details are now reported, including studies on solutions of pure pisatin and plant extracts (diffusates) containing pisatin.

(i) *Recrystallized Pisatin*.—Pisatin contains only carbon, hydrogen, and oxygen and has the molecular formula $C_{17}H_{14}O_6$. A preliminary report on the structure of pisatin has been given elsewhere (Perrin and Bottomley 1961). The characteristic ultraviolet absorption spectrum, λ_{\max} 286 m μ ($\log \epsilon$ 3.68) and 309 m μ ($\log \epsilon$ 3.86) in ethanol, has formed the basis of the quantitative chemical assay described above and has been used in investigations of chemical and physical characteristics of pisatin.

Distribution coefficients for pisatin have been determined between light petroleum (b.p. 55–60°C) and water (2.3 : 1), cyclohexane and water (3.6 : 1), and between oleyl alcohol and water (100 : 1). Oleyl alcohol was selected as representative of fatty acids found in cytoplasmic membranes (Albert and Hampton 1954).

Pisatin is stable in neutral and alkaline solutions. There is no change in the ultraviolet absorption spectrum even in 0.05M sodium hydroxide. This fact, together with its ready extractability from alkaline solutions, indicates the absence of phenolic or other acidic groups. Pisatin is, however, extremely acid labile, forming a compound which has been recrystallized and which analyses as $C_{17}H_{12}O_5$. This substance contains one molecule of water less than pisatin, and has been designated anhydropisatin (Perrin and Bottomley 1961). The dehydration of pisatin is accompanied by a considerable bathochromic shift and intensification of the ultraviolet absorption spectrum with maxima at 339 m μ ($\log \epsilon$ 4.58) and 358 m μ ($\log \epsilon$ 4.60). The quantitative formation of anhydropisatin from pisatin as measured at these maxima, has been used to confirm the identity of pisatin and the presence of low levels of this substance in the chemical assay described above.

The solubility of pisatin at 23°C is 0.03 mg/ml in water, 0.5 mg/ml in light petroleum, 6 mg/ml in oleyl alcohol, and >42 mg/ml in ethanol.

(ii) *Plant Extracts containing Pisatin*.—In extracts or diffusates from infected plant tissues, pisatin occurs in a biologically active state at concentrations of the order of two to three times its maximum solubility in distilled water. Experiments were undertaken to study the stability of pisatin in such a system with the following results. The level of pisatin in a diffusate solution (pisatin concentration 61.3 μ g/ml) was not lowered as a result of autoclaving (15 lb for 20 min), heating to 96–98°C for 30 min, or heating to 65–70°C for 15 hr. There was no sedimentation of pisatin from the diffusate by centrifuging at 3200 g for 30 min, but there was a slight loss (c. 3%) following centrifuging at 18,400 g for 30 min. Filtration through a "Millipore"* filter of mean pore size 0.45 μ removed 14% of the pisatin from the diffusate, while the pisatin content of a filtrate from a 0.01 μ pore size filter was close to 30 μ g/ml, i.e. the value for the solubility of pisatin in distilled water. Biological activity paralleled the physical measurements of pisatin.

Pisatin is adsorbed on to or dissolves in certain plastic surfaces such as polyvinyl chloride or cellulose nitrate plastics, when the plastic surfaces are brought in contact with diffusate solutions. Pisatin is also strongly adsorbed on to many chromatographic adsorbents such as celite, lactose, sucrose, calcium carbonate, alumina, and charcoal, but it can be removed fairly readily from celite, the sugars, and calcium

* Manufactured by Millipore Filter Corporation, Watertown, Massachusetts.

carbonate with aqueous ethanol. The adsorption or loss of pisatin on to plant surfaces from saturated aqueous solutions and from diffusates with a similar concentration of pisatin ($30.3 \mu\text{g/ml}$) were studied by placement of drops of the solutions on pea and tobacco leaf surfaces and on the endocarp tissue of pea pods. The plant tissues were incubated at 20°C in the dark for 24 hr, the solutions withdrawn from the surfaces, and the pisatin concentration measured. No loss of pisatin occurred on to the leaf surfaces. The mean loss (four replicates) from the solutions placed on endocarp tissues was 41% for the diffusate solution and 62% for the solution of pure pisatin respectively.

TABLE 2
EFFECT OF LIGHT ON PISATIN SOLUTIONS

Light Treatment	Wavelength ($\text{m}\mu$)	Period of Treatment (min)	Pisatin Concn. ($\mu\text{g/ml}$)
Untreated			61.3
Ultraviolet light	365	120	54.3
Sunlight (approx. 5000 f.c.)		180	26.3*
Ultraviolet light	253.7	15	57.4
Ultraviolet light	253.7	30	53.4
Ultraviolet light	253.7	45	49.5*
Ultraviolet light	253.7	60	48.2*
Ultraviolet light	253.7	90	44.2*

* No antifungal activity detected.

To study the effect of light, including ultraviolet, samples of diffusate (pisatin concentration $61.3 \mu\text{g/ml}$) were subjected to various types of irradiation and the residual pisatin concentration measured. The results, presented in Table 2, indicate that the previously established correlation between biological activity and pisatin concentration was not valid after the sunlight and $253.7 \text{ m}\mu$ irradiations. It was found that the pisatin-like compound remaining in the irradiated solutions did not dehydrate readily under mild acid conditions with the formation of anhydropisatin. Careful examination of the ultraviolet spectrum of samples following irradiation treatment showed a gradual change to a substance which is spectroscopically similar to pisatin (λ_{max} , $286 \text{ m}\mu$ and $311 \text{ m}\mu$ but without the inflexion at $281 \text{ m}\mu$). It is suggested that this substance is chemically close to pisatin but that its biological activity has been changed by the high-energy ultraviolet irradiation.

(h) Biological Properties of Pisatin

The nature of the antifungal activity of pisatin was studied in two types of experiment. In the first, drops of freshly collected diffusate containing spores of *M. fructicola* were taken from a standard inoculation experiment (pisatin concn. $60 \mu\text{g/ml}$). The germinated spores, whose growth had been stopped by the pisatin in the diffusate, were removed from the diffusate by centrifugation and resuspended in sterile distilled water. Single drops of the latter were placed on slices (2.5 cm dia.,

1 cm thick) of banana mesocarp, which had previously been shown to be highly susceptible to *M. fructicola*, under aseptic conditions in petri dishes and incubated for 5 days at 20°C. Nine replicates were used, with sterile water serving as control. Abundant sporulation occurred on all banana slices to which the drops of spore suspension had been added. No sporulation occurred on the controls. In the second test, six seeded agar disks (prepared as for bioassay) were incubated in a pisatin solution in 5% ethanol (pisatin concn. 118 µg/ml) for 48 hr. No germination occurred. The seeded agar blocks were then removed from the pisatin solution and washed in three changes of distilled sterile water. Finally the blocks were reincubated in sterile water for a further 24 hr at 20°C. The resultant mean percentage germination was 94.8%. However, germ tubes were short, distorted, and branched. The results of these tests show that pisatin possesses fungistatic rather than fungicidal activity.

Hypersensitivity in plants is normally associated with necrosis of infected host cells. The following experiments were carried out to determine if pisatin at concentrations normally present in diffusates is phytotoxic. In the first test, drops of diffusate (pisatin concn. 75.2 µg/ml) were placed on proliferated endocarp cells of the pea pod and left for 48 hr. The viability of the cells under the drops was determined using 0.1% neutral red (Tribe 1955). In the second test, tobacco seedlings and young pea plants were sprayed to incipient runoff with diffusate (pisatin concn. 65.8 µg/ml). The plants were incubated at high humidity for 24 hr in the dark at 18°C and then returned to the glass-house. They were examined for phytotoxicity symptoms 24 hr, 48 hr, and 1 week after treatment. In neither of these experiments was any evidence obtained to suggest that pisatin was toxic to pod cells or leaf tissues.

A third experiment was carried out to determine the effect of pisatin on growth. Ten pregerminated wheat seeds (coleoptiles approx. 1 mm in length) were placed in a petri dish containing 3 ml of one of the following solutions: diffusate (pisatin concn. 65.8 µg/ml), diffusate residue (pisatin extracted), and distilled water. Ten dish replicates were used. The seeds were incubated at 20°C and the mean lengths of the primary roots (radicles) after 48 hr were 21.51, 37.58, and 31.77 mm, respectively. Difference for significance at the 0.1% level was 5.48 mm. The results show that pisatin inhibited the growth of the primary root by one-third if the length in distilled water is taken as the reference length.

III. DISCUSSION

Allen's (1959) primary conclusion regarding the nature of the plant's defence against pathogenic attack was that "it is not a condition of the plant which constitutes resistance but a process of response". This concept of a response or interaction between the host and parasite with the formation of new antifungal compounds is the basic concept in the phytoalexin theory postulated by Müller and Börger (1940) on the basis of important indicative evidence. As a part of this response or interaction respiration increases in diseased tissues (Millerd and Scott 1956; Shaw and Samborski 1957), and biochemical changes occur. Changes in normal metabolites have been reported (Kuć, Ullstrup, and Quackenbush 1955) but the compounds concerned were not adequate in themselves to explain in full the inhibition of growth of the invading fungus. Three previous reports have presented data on the isolation of abnormal

metabolites, namely ipomeamarone (Hiura 1943) formed in sweet potato tissues infected with *Ceratostomella fimbriata* (Ell. & Hals.) Elliot, orchinol (Boller *et al.* 1957) which results from the interaction between *Rhizoctonia repens* Bern. and orchid tubers, and an antifungal compound (Condon and Kué 1960) isolated from carrot root tissue infected with *Ceratostomella fimbriata*, which may be considered to be formed as a "process of response" to fungal infection. In these reports, however, the preparation of the host tissue involved extensive cell damage, and the conditions and duration of the incubation of the tissues after inoculation in the second and third examples might cast reasonable doubt on the primary nature of the compounds isolated. Ipomeamarone was originally thought to be primarily an uncoupling agent; however, phytoalexin action has recently been envisaged for it at the higher concentration levels (Akazawa 1960).

Pisatin is formed as a response to inoculation of the endocarp tissues with *M. fructicola*. The suggestion that pisatin is the primary antifungal compound formed as a response of pea pod endocarp tissues to inoculation with *M. fructicola* results from the following observations. There is a close association between the hypersensitivity symptoms of the inoculated endocarp tissues, the limited growth of the fungal germ tubes in the inoculum at the end of the incubation period, and the concentration of pisatin in the resultant diffusate. The technique involves a minimum of damage to host cell tissues. The incubation period required for the formation of inhibitory levels of pisatin is relatively short. The drop-diffusate technique takes advantage of the plant cell membrane as the primary "filter" in the extraction procedure. The simplicity of the subsequent extraction and purification, which utilizes only light petroleum and ethanol, is strong evidence that pisatin occurs naturally in infected tissues, and is not an artefact of our extraction technique, as has been shown to be the case in some recent papers reporting the isolation of some other antifungal compounds (Virtanen and Hietala 1960). Thus it would appear that pisatin would qualify as an example of the biologically active class of compounds defined by Müller (1953) as "phytoalexins".

Some of the general properties of pisatin have been studied in this paper. When these are compared with those reported by Müller (1958) for the phytoalexin from French beans (*Phaseolus vulgaris* L.) it will be seen that pisatin is similar in some of its properties. Both are lipophilic in so far as they can be extracted into light petroleum. Solutions of both are stable to high temperatures (*c.* 100°C). Solutions of pisatin are stable to autoclaving (15 lb, 20 min). The compounds differ, however, in their stability to light. The French bean phytoalexin (Müller 1958) rapidly loses biological activity on exposure to ultraviolet light ($\lambda = 365 \text{ m}\mu$). This radiation has only a slight effect on the biological activity of pisatin even after extended exposure (2 hr). Irradiation by sunlight or ultraviolet light ($\lambda = 253.7 \text{ m}\mu$), however, rapidly destroys the biological activity of pisatin (*cf.* Uehara 1958c).

The low solubility of pisatin in aqueous solutions has been a major problem in the study of its biological properties. A similar problem has been discussed by Gaumann and Kern (1959) where it is stated that the concentration of orchinol in water at pH levels characteristic of those of the host tissues is roughly 10 times too low to produce inhibition areas of similar size to those produced by infected tuber

fragments. In our results, it is shown that the saturation level of pisatin in water is approximately $30\mu\text{g/ml}$. However, the concentration of pisatin in the diffusates is normally two to three times this concentration. Gaumann and Kern (loc. cit.) explained the discrepancy in their solubility results by suggesting the existence of a solubilizing agent which keeps orcinol in excess of saturation. The results presented above provide strong evidence that pisatin exists in diffusates both in true solution and in a fairly stable dispersed form in which it is biologically active. High adsorption was reported by Müller (1958) as an important character of French bean phytoalexin. Pisatin, as discussed above, occurs in diffusates in excess of its maximum solubility in water. Under such conditions it is lost readily from solution on to various types of surfaces. The adsorption characteristics of pisatin in true solution are, however, quite normal. The drop in pisatin concentration in samples placed on endocarp tissues could be adsorption; on the other hand it could be due to destruction by enzymatic activity. No evidence is at present available on this point.

Müller (1958) stated that French bean phytoalexin was fungistatic at lower concentrations and fungicidal at the higher levels. Pisatin at concentrations equivalent to the maximum concentrations obtained in diffusates has been shown to be only fungistatic, and it is only by the maintenance of the pisatin concentration at inhibitory levels that growth of the fungus is prevented. Resistance, and in particular the hypersensitive reaction in plants, is characterized by the necrosis of host cells surrounding the infection site. This type of reaction is clearly demonstrated in the work of Müller (1958) on French beans. A similar, but less intense necrotic response occurs in peas. The response varies in peas but is not generally apparent over the inoculated areas until 72 hr after inoculation. Moreover it occurs more rapidly if the inoculum liquid is removed after 40 hr than if it is left on the tissue surfaces. The results of phytotoxicity tests suggest that mature cells are not sensitive to pisatin, but that pisatin has a significant effect on rapidly dividing cells, and thus affects rate of growth (cf. acti-dione (Hawthorne and Wilson 1952)). Thus, pisatin is phytotoxic, although there would appear to be a fairly large concentration differential between the equivalent effect of pisatin on fungi and on green plant cells. It may account for the necrosis of host cells *in vivo*, but at this stage of our work the evidence in favour of this is not very strong.

Detached pods were used in the bulk extraction of pisatin; however, pisatin formation is not limited to pod tissues. Its isolation from inoculated pea leaf, stem tissue, and pods on the growing plant indicate the wide distribution of the capacity to form pisatin in pea plant tissues, and suggest that the results obtained in terms of the pod tissues are of pathological significance in relation to the whole pea plant.

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THE GROWTH OF *RHIZOBIUM* IN SYNTHETIC MEDIA

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Summary

A chemically defined medium for the growth of *Rhizobium* is described in which populations of up to 5×10^9 cells/ml were obtained. For the six strains of bacteria studied the complete medium supported exponential growth for two to five generations. The concentrations of biotin giving best growth varied with strain between 125 and 250 $\mu\text{g/l}$ when the nitrogen source was sodium glutamate. NH_4^+ , NO_3^- , and other amino acids, singly or in combination, did not support as good growth as did sodium glutamate.

The medium was used to demonstrate a calcium requirement for growth of all strains. When the concentration of this ion was reduced to 0.1 p.p.m. with four strains, lag was lengthened and the exponential phase was shortened but growth rate was not affected. With two other strains, calcium deficiency did not affect lag or the length of the exponential phase, but the growth rate was reduced. Calcium-deficient cultures attained final populations which were 20–60% lower than those grown in the complete medium, and with four strains deficient cells were enlarged.

I. INTRODUCTION

Monod (1949) stated "the study of the growth of bacterial cultures does not constitute a specialized subject or branch of research: it is the basic method of microbiology". This basic study of *Rhizobium* has been very largely neglected as most published work has been concerned with total populations and the ability to survive serial subculture rather than with the dynamics of growth.

West and Wilson (1940) and Wilson and Wilson (1942) used serial-subculture methods to show that biotin may be essential, stimulatory, or without effect for the growth of *Rhizobium*. More recently Norris (1959) was able to subculture the organisms for prolonged periods in media containing less than 0.1 p.p.m. calcium, and concluded that they were satisfied with minute traces of this element. In this work Norris used both a yeast extract-mannitol medium and a synthetic medium. Bergersen (1958) obtained linear growth of *Rh. japonicum* in yeast extract-mannitol medium; that is, the generation time of the bacteria became progressively longer and the steady state of growth was not attained. Preliminary use of Norris' (1959) synthetic medium in this Laboratory showed that this medium supported only a brief exponential phase of growth after a lag lasting in most cases several days. These results suggested that these two media used in Norris' (1959) work were severely growth restrictive and thus it seemed that the omission of the Ca^{++} ion could not be expected to have much effect when the growth of the bacteria was already restricted by the constitution of the media.

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The objective of the work described in this paper was, therefore, the development of a synthetic defined medium for the growth of *Rhizobium*, which would support a considerable period of exponential growth after as short a lag as possible and give a high density of total growth in as short a time as possible. The medium was then used to re-examine the effects of calcium deficiency upon the growth of *Rhizobium*.

II. MATERIALS AND METHODS

(a) Bacterial Strains

Six strains were used throughout, four of them being among those used by Norris (1959): Rothamsted AH₂, CCI46A (*Rh. meliloti*); Rothamsted CIF, SU297/3 (*Rh. trifolii*); CB170 (*Rhizobium* sp. isolated from *Phaseolus lathyroides*); CB362 (*Rhizobium* sp. isolated from *Psoralea eriantha*). These were maintained on yeast extract-mannitol agar in screw-capped bottles. For determination of growth curves the organisms were grown in tubes of the medium to be used so that, with the comparatively large inocula used, there would be no carry-over of nutrients not present in the test medium. The volumes of these tube cultures used to inoculate the growth flasks were adjusted from counting-chamber counts so that the same number of organisms was used in inoculating all flasks of an experiment.

(b) Growth Media

Yeast extract-mannitol medium was that used by Nutman (1946), omitting the agar when a liquid medium was required.

The salts of the synthetic medium were basically the same as those used by Norris (1959): Na₂HPO₄·12H₂O, 0·045%; MgSO₄·7H₂O, 0·01%; FeCl₃, 0·002%, and CaCl₂, 0·004%, were added to the medium as separately sterilized solutions after autoclaving. The energy source was 1% mannitol. All media were adjusted to pH 6·8.

In the determination of the best nitrogen source, KNO₃, NH₄H₂PO₄, glutamic acid (sodium salt), asparagine, aspartic acid, arginine, histidine, tyrosine, lysine, and serine were all used singly or in combination to give 8 mg nitrogen/100 ml medium. For this work 100 µg thiamine and 25 µg biotin were used per litre of medium.

Biotin requirements were determined in media containing the above salts and mannitol with sodium glutamate as the nitrogen source and 100 µg thiamine/l. Biotin was obtained from Hoffmann la Roche and Company, Basle, and was made up in pH 7·0 phosphate buffer and stored at 4°C.

In the experiments to determine the effects of calcium deficiency the constituents were purified. Mannitol was three times recrystallized from supersaturated solutions. MgSO₄ was recrystallized from a filtered supersaturated solution and the crystals washed with alcohol. Sodium glutamate was dissolved in water and precipitated as the acid by the addition of conc. HCl; the acid was washed with cold water and then suspended in water and titrated to neutrality with high purity NaOH. The solution was then poured into six volumes of ethanol and crystallized at 4°C overnight. The crystals were washed with cold ethanol and dried. Na₂HPO₄ was used without further purification and glass-distilled water was used throughout. The calcium levels, determined by an atomic absorption spectrophotometric method, are given for the various constituents in Table 1, which shows that although they contain

a total of less than 0.088 p.p.m. calcium in the concentrations used in the medium, calcium leached from the glass during autoclaving raised the level to 0.11 p.p.m. All glassware was soaked in 25% HCl overnight and then exhaustively washed with glass-distilled water before use: glassware used for low-calcium cultures was not used for plus-calcium cultures. Cotton wool and gauze plugs were prepared from materials washed in 2% ethylenediaminetetraacetic acid (EDTA) and distilled water.

TABLE I
RESIDUAL CALCIUM IN PURIFIED CONSTITUENTS OF SYNTHETIC MEDIUM

Purified Constituent	Concentration of Solution Analysed	Calcium Content (p.p.m.)	Calculated Calcium in Medium Used (p.p.m.)
Mannitol	10%	<0.05	<0.005
MgSO ₄ ·7H ₂ O	0.1%	0.09	0.009
Na ₂ HPO ₄ ·12H ₂ O	0.45%	<0.05	<0.005
Sodium glutamate	1.1%	0.62	0.062
FeCl ₃	0.02%	0.05	0.005
Biotin	25 µg/ml	<0.05	<0.005
Thiamine	1 mg/ml	0.14	0.0014
Total			<0.0879
Low-calcium medium analysed after autoclaving			0.11

(c) Growth Conditions

Cultures were grown in 250-ml Erlenmyer flasks which were equipped with $\frac{1}{2}$ -in. diameter side-arms for turbidity measurement: 40 ml of medium was used per flask. Inocula were 1–3 ml of young tube cultures and the flasks were incubated at 25°C on a rotary shaker running at 160 c/min.

(d) Measurement of Growth

Growth was measured turbidimetrically in an "EEL" photoelectric nephelometer. Total numbers of bacteria were determined from calibrations prepared for each strain of bacteria in each medium used, by measurement of the turbidity of suspensions which had been counted in a Petroff–Hauser counting chamber.

The various growth characteristics were determined graphically by plotting log₂ bacterial numbers against time (see Fig. 1); this facilitated determination of growth rate, since an increase of 1 unit was equivalent to a doubling of the population (Monod 1949). These growth characteristics were:

- (i) Lag time (L), the time between inoculation and the commencement of growth had all cells commenced to divide together at the maximum rate (Hinshelwood 1946);
- (ii) The generation lag (L_g), the time for the first doubling of the initial population (Lockhart 1960);

- (iii) The growth rate in the steady (exponential) state in generations per hour (R);
- (iv) The duration of the exponential phase (E);
- (v) The total population increase during the growth period (G).

One strain, SU297/3, grew in small uniform clumps and the characteristics of growth were determined from turbidity alone.

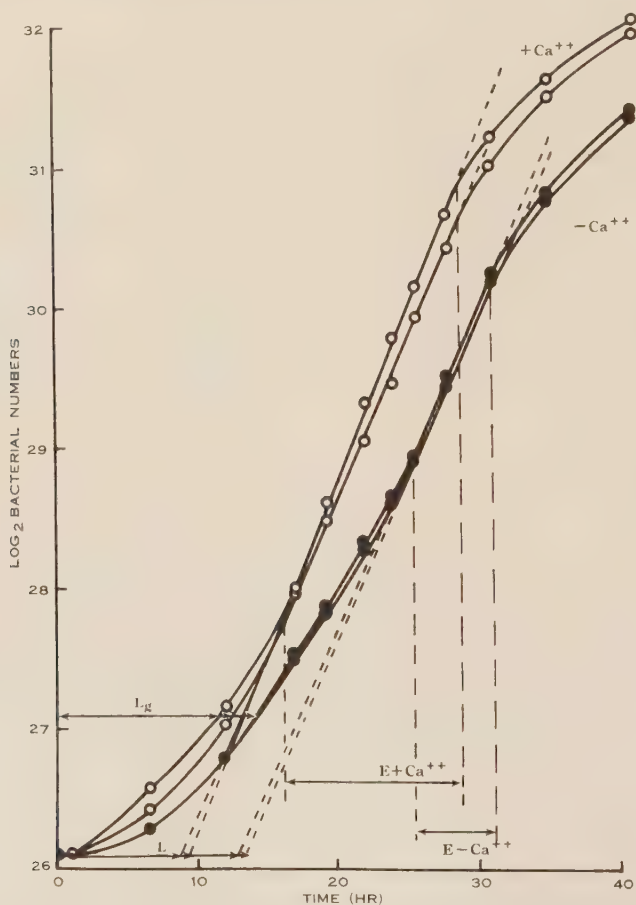


Fig. 1.—Determination of the growth characteristics of *Rhizobium*.
Data for duplicate flasks are plotted for strain AH₂.

(e) Plant Tests

Tests of infectiveness and symbiotic effectiveness were done as follows in order to compare the synthetic medium with the commonly used yeast extract-mannitol medium. Cultures were carried through two subcultures in tubes followed by growth in shake flasks after which single colonies from the same medium, solidified with agar and spread with the flask culture, were used to inoculate appropriate host plants. After growth in a glass-house for 5 weeks the plants were harvested, nodulation examined, and dry weights obtained.

III. EXPERIMENTAL RESULTS

(a) Nitrogen Source

Representative growth curves for strain ClF grown in the salts-mannitol medium with 25 μg biotin and 100 μg thiamine per litre and using various nitrogen sources are illustrated in Figure 2. The results of these studies clearly showed that glutamate was the best source of nitrogen for growth of all strains used. In addition

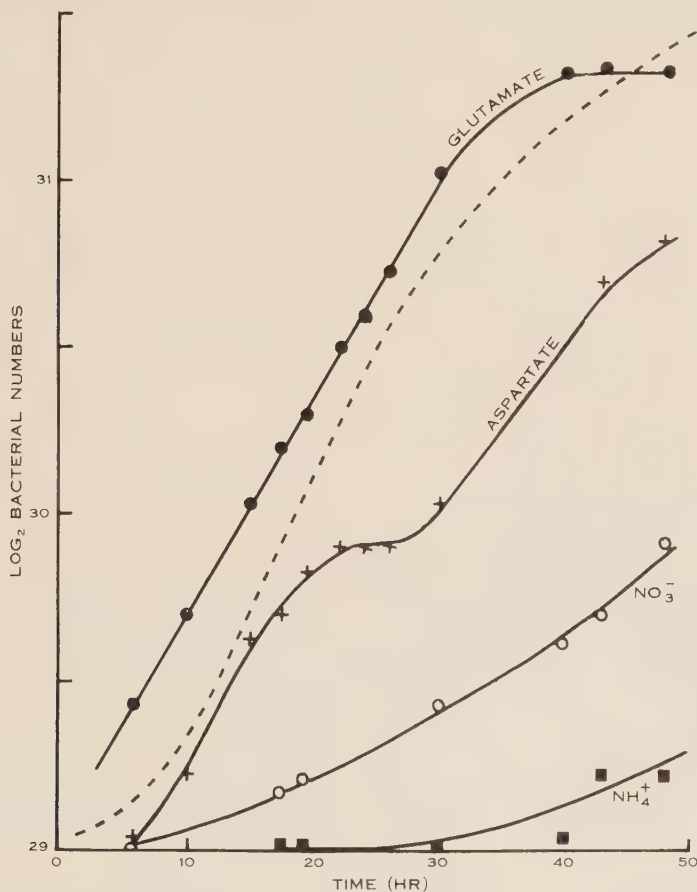


Fig. 2.—Effect of various nitrogen sources upon the growth of strain ClF (*Rhizobium trifolii*) in shake culture. The broken line shows the growth of this strain in yeast extract-mannitol medium from a separate experiment. The growth curve labelled NO_3^- is the curve for the medium used by Norris (1959).

to the results illustrated, it was found that no combination of amino acids or amino acids and inorganic nitrogen was as good as glutamate. Amino acid mixtures frequently gave multi-step growth curves of the type shown for aspartate, but with several steps. The reasons for this are not known and although high total numbers were often attained with these cultures the forms of the growth curves were quite unsuited to growth studies.

The use of the sodium salt of glutamic acid as a nitrogen source resulted in general in a greater rise in pH after growth in the synthetic medium than after growth in yeast extract-mannitol medium (Table 2).

(b) *Biotin Requirements*

Preliminary results indicated that the 0.5 $\mu\text{g/l}$ used by Norris (1959) was sub-optimal and hence 25 $\mu\text{g/l}$ was used in the tests of nitrogen sources. A more extensive investigation was then carried out using glutamate as nitrogen source. The results of this are summarized in Table 3, which shows that strain CC146A had no exponential

TABLE 2

pH OF THE CULTURES, MEASURED WITH A GLASS ELECTRODE, AFTER 72 HR. INITIAL pH 6.8

Bacterial Strain	Synthetic Medium	Yeast Extract-Mannitol Medium	Bacterial Strain	Synthetic Medium	Yeast Extract-Mannitol Medium
AH ₂	7.75	7.1	CB362	7.1	7.05
CC146A	7.6	6.9	CIF	8.1	7.4
CB170	7.7	7.2	SU297/3	7.6	6.6

phase with only 12.5 $\mu\text{g/l}$ biotin and strain SU297/3 required more than 25 $\mu\text{g/l}$ for exponential growth. All other strains grew exponentially at all biotin concentrations used, but total growth increased with increased biotin up to 125–250 $\mu\text{g/l}$ according to strain. Biotin did not affect growth rate (R) but the length of the exponential phase (E) was increased with raised biotin levels for most of the strains. Both L and L_g were either unaffected or reduced with increased biotin except for strain AH₂ for which levels higher than 125 $\mu\text{g/l}$ were inhibitory with respect to L , E , and G . With all strains raised biotin levels prolonged growth after the end of the exponential phase and the rate of retardation was greatest at the lowest biotin concentrations.

(c) *Effects of Low Calcium*

The effects on growth of the omission of calcium from the synthetic medium, using glutamate and supplying the best biotin concentration for each strain, are summarized in Table 4. Preliminary tests showed that 0.02% CaCl_2 as used by Norris (1959), was slightly inhibitory for strain AH₂ so the plus-calcium series contained 0.004% CaCl_2 or 14 p.p.m. calcium, while the low-calcium series contained about 0.1 p.p.m. calcium.

For four strains, AH₂, CC146A, CB170, and CIF, both L and L_g were increased in calcium-deficient cultures and the exponential phase (E) was shortened, although R was not affected. With the other two strains there was little effect on L or L_g and E was not affected by low calcium but the growth rate (R) was reduced. With all

strains calcium deficiency was expressed as a reduction in total growth, G varying from 40–80% of that attained with added calcium.

Determination of the level of calcium required proved difficult firstly because it was not possible to reduce the calcium content of the unautoclaved medium below

TABLE 3

EFFECTS OF BIOTIN CONCENTRATION ON GROWTH OF RHIZOBIUM IN SHAKE CULTURE

L = lag phase (Hinshelwood 1946) in hr; L_g = generation lag in hr; R = growth rate in generations per hr in exponential phase; E = length of exponential phase in hr; G = bacterial increase $\times 10^7$

Bacterial Strain	Biotin ($\mu\text{g/l}$)	L	L_g	R	E	G^*	Growth Period (hr)
AH ₂	25	4.5	8.3	0.26	13.5	236	41
	125	5.0	8.8	0.26	18.25	532	41
	250	6.0	9.5	0.26	13.0	492	41
	375	7.5	9.8	0.26	10.5	470	41
CC146A	12.5	—†	4.0	—†	0	72	54.5
	25	0.75	5.5	0.21	23.0	122	54.5
	125	0	4.5	0.21	24.0	308	54.5
	250	0	4.5	0.21	24.0	320	54.5
CB170	12.5	34.0	45.0	0.05	33.0	122	127
	25	29.0	44.0	0.05	49.0	153	127
	250	21.0	41.0	0.05	58.0	169	127
CB362	12.5	3.3	9.5	0.16	24.0	86	54.5
	25	7.0	13.0	0.17	27.6	154	54.5
	125	7.0	13.0	0.17	27.6	233	54.5
	250	7.0	13.0	0.17	27.6	228	54.5
CIF	12.5	4.5	12.0	0.13	19.0	60	53.5
	25	4.5	12.0	0.13	20.6	74	53.5
	125	4.5	12.0	0.13	23.5	88	53.5
	250	4.5	12.0	0.13	23.5	85	53.5
SU297/3	25	—†	3.8	—†	0	c. 400‡	41
	125	3.5	8.5	0.20	19.5	c. 600	41
	250	2.5	7.5	0.20	19.5	c. 600	41
	375	1.5	6.5	0.20	19.5	c. 600	41

* Includes growth in the phase of retardation: stationary phase was reached only in the biotin concentrations 12.5 and 25 $\mu\text{g/l}$.

† All growth in phase of retardation.

‡ Approximate numbers only because of clumpy growth of this strain.

0.08 p.p.m., and secondly because of variable amounts of calcium leached from the growth flasks during autoclaving, no satisfactory concentration series was obtained. However, for the strains of *Rhizobium* tested a response to 5 p.p.m. added calcium

could be detected and the maximum response occurred between 10 and 14 p.p.m. No treatment of the glassware prevented calcium leaching during autoclaving, although boiling and autoclaving with EDTA solutions, detergent, and acid treatments were tried. The 25% HCl soaking was slightly better than other treatments. Another

TABLE 4
EFFECTS OF CALCIUM UPON GROWTH OF RHIZOBIUM IN SHAKE CULTURE

Bacterial Strain	Calcium*	L †	L_g †	R †	E †	G †	Growth Period (hr)
AH ₂	+	9.0	11.75	0.25	12.5	437	41
	+	8.75	12.5	0.24	12.5	413	41
	—	12.0	17.0	0.24	5.5	275	41
	—	12.0	17.0	0.22	5.5	269	41
CC146A	+	0	6.0	0.19	25.0	263	41
	+	0.6	6.6	0.16	28.0	288	41
	—	4.0	8.0	0.18	7.0	220	41
	—	6.0	8.0	0.18	7.0	228	41
CB170	+	10.0	27.0	0.05	40.0	180	96
	+	14.0	32.0	0.05	42.0	165	96
	—	29.0	44.0	0.05	17.5	108	96
	—	29.0	47.0	0.05	17.5	105	96
CB362	+	8.0	14.6	0.15	27.0	253	54.5
	+	8.0	15.5	0.13	25.0	218	54.5
	—	7.0	17.0	0.10	24.0	140	54.5
	—	9.6	19.0	0.11	26.0	145	54.5
C1F	+	5.3	12.6	0.14	20.6	85	50.5
	+	5.3	12.6	0.14	20.6	82	50.5
	—	8.5	16.5	0.13	12.3	32	50.5
	—	8.6	17.0	0.13	12.3	33	50.5
SU297/3	+	2.0	8.0	0.17	23.0	c. 450‡	41
	+	2.5	8.5	0.17	23.0	c. 450	41
	—	1.0	8.25	0.13	24.0	c. 250	41
	—	2.0	8.5	0.13	23.0	c. 250	41

* + = medium contains 14 p.p.m. calcium; — = medium contains 0.1 p.p.m. calcium.

† L , L_g , R , E , and G as in Table 3.

‡ Approximate numbers only, because of the clumpy growth of this strain.

difficulty encountered here was that of the analysis of low levels of the ion. Concentration of the medium was required and this also introduced the possibility of calcium being introduced from the glassware. With care, however, it was possible to demonstrate depletion of calcium from plus-calcium medium analysed before and after growth of the bacteria.

Another effect of calcium deficiency appeared when the growth flasks were calibrated for turbidity with suspensions of known numbers of cells from plus- and minus-calcium media. Figure 3 shows one such calibration. The difference in slope is attributable to the increased size of the deficient cells, which was readily seen when

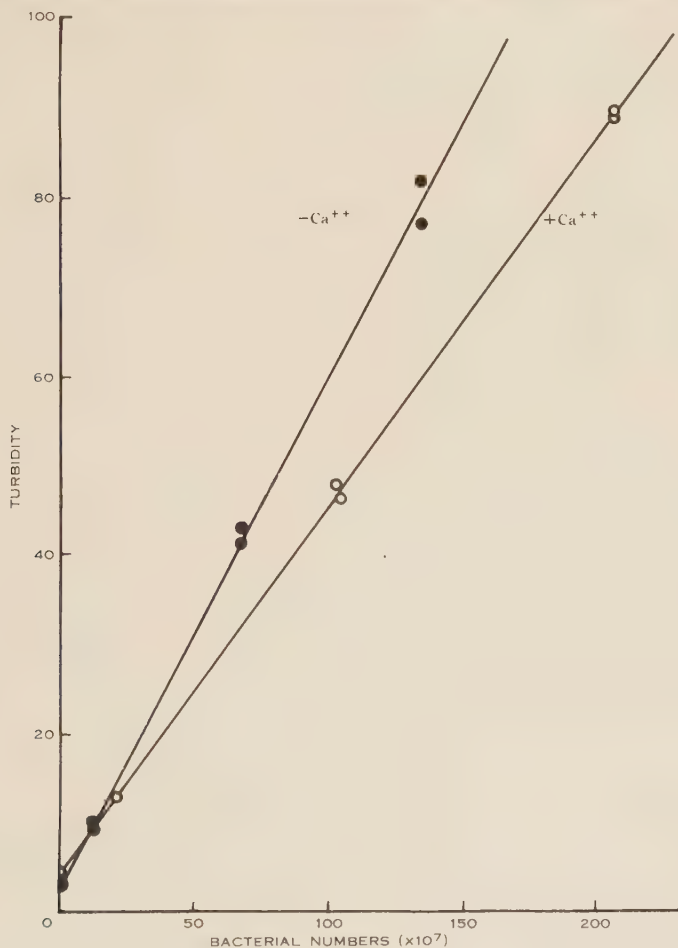


Fig. 3.—An example of the turbidity *v.* cell numbers calibration in which the different slope is a measure of difference in cell size. Strain CB362.

counting the suspensions in the Petroff-Hauser chamber. Strain CC146A did not show this difference in size and strain SU297/3, because of the clumpy growth, could not be assessed for cell size differences.

(d) *Synthetic Medium Compared with Yeast Extract-Mannitol Medium*

The growth of the six strains in yeast extract-mannitol medium and in the synthetic medium was compared. Strain CB170 grew better in the complex medium but the others grew as well in terms of *L*, *R*, and *G* in the synthetic medium as they

did in yeast extract-mannitol medium: *E* was longer for growth in the synthetic medium.

Strains AH₂, SU297/3, CC146A, and ClF grew better on synthetic medium solidified with 2% agar than on yeast extract-mannitol agar when these media were used for the isolation of single colonies for the plant tests.

The growth of ClF in yeast extract-mannitol medium is shown for comparison with the synthetic medium as a broken line in Figure 2.

(e) *Symbiotic Properties after Growth in Synthetic Medium*

With the exception of strain ClF, single colonies of all strains did not differ in infective properties or in symbiotic effectiveness, whether grown in the synthetic medium or in yeast extract-mannitol. Strain ClF, however, lost some degree of infectiveness, only 2 colonies out of 10 from the synthetic medium producing nodules on white clover while 6 out of 10 from the yeast extract-mannitol medium nodulated the host. With the other strains, all colonies tested from both media produced effective nodules on appropriate hosts.

(f) *Conclusions*

A synthetic growth medium for *Rhizobium*, which gives high total populations and in which growth characteristics are suitable for dynamic studies, is constituted as follows: mannitol 1%; Na₂HPO₄·12H₂O, 0·045%; MgSO₄·7H₂O, 0·01%; FeCl₃, 0·002%, and CaCl₂, 0·004%, added after autoclaving; sodium glutamate 0·11%; thiamine 100 µg/l; biotin 125–250 µg/l, depending on bacterial strain.

With the exception of strain CB170, the synthetic medium produced as good growth, in terms of maximum populations, as yeast extract-mannitol medium, but in much shorter growth periods and with relatively longer phases of exponential growth.

The synthetic medium, solidified with 2% agar, was superior to yeast extract-mannitol for strains AH₂, SU297/3, CC146A, and ClF.

All strains of *Rhizobium* studied exhibited a response to added calcium when the concentration of that ion in the medium was about 0·1 p.p.m. This response was manifested in the following ways:

- (i) Low-calcium media gave longer lag (*L* or *L_g*) and a shortened exponential phase, or
- (ii) Lag was not affected but growth rate in the exponential phase was reduced.
- (iii) Low-calcium media produced only 40–80% of the growth with added calcium.
- (iv) Calcium-deficient cells of four strains were larger than those from media with added calcium.

IV. DISCUSSION

In discussing the results presented in this paper, attention is first of all drawn to the use of glutamate as a nitrogen source for the growth of *Rhizobium*. Yeast extract, as used in non-defined media, supplies both growth factors and nitrogen as amino acids. Glutamate is known to stimulate vigorous respiration of *Rhizobium* (Bergersen 1957). Other workers have chosen an inorganic ion such as nitrate as a

source of nitrogen for a synthetic medium (Norris 1959). In its natural location in the soil *Rhizobium* grows predominantly in plant rhizospheres where root exudates, especially of legumes, provide relatively large amounts of amino acids (Rovira 1956).

The unusually high biotin requirements which have been demonstrated may be linked with the use of glutamate as nitrogen source, which permits so much more rapid growth than occurs with nitrate. Alternatively, the biotin may be a source of some other growth factor present as an impurity or may be serving as a source of a fatty acid from the side-chain of the molecule. Thiamine was stimulatory for only two strains and its inclusion in all media was a step towards uniformity. Once again it may be stated that biotin is a significant constituent of root exudates of legumes although absolute amounts may be small, and is thus of importance for growth of *Rhizobium* in the rhizosphere of host plants (Rovira and Harris 1961).

It is not suggested that the synthetic medium used in this work is the best possible but the main objective has been to emphasize the principles which should be observed in such studies. The salts used by Norris (1959) are apparently satisfactory but nitrate is not a good nitrogen source. Glutamate is shown to be better for the six strains studied, but with other strains other amino acids may be better and give more satisfactory growth. Similarly, biotin requirements are likely to be different for other strains and the level of this vitamin in the medium should be that best suited to the strain being studied. A good growth medium should fulfil the following conditions:

- (i) The lag should be as short as possible so that the original character of the culture is not altered by the selections of mutants.*
- (ii) The exponential phase should be as long as possible with the best growth rate attainable. This is the critical stage of growth since it is in the exponential phase that the steady state is reached and thus it is the phase in which experimental deficiencies are most likely to be expressed.
- (iii) The maximum population should be as high as possible if the medium is to be of any practical importance.

The growth characteristic L_g was included in this study for the reasons stated by Lockhart (1960), viz. it seems to be a better measure of lag when different growth rates are involved. With the six strains used it has been possible to test Lockhart's (1960) hypothesis that L_g is very close to the time that lag actually ends; in other words, when all the cells of the initial culture have divided once, all cells should be growing at the maximum rate. Strains which had a lag phase varied in their relation between L_g and onset of exponential growth, but in most cases with the complete synthetic medium, L_g was quite close to the time at which the steady state commenced. Calcium deficiency increased the discrepancy by delaying the onset of exponential growth for up to three generations in four of the six strains studied.

The other effects of calcium deficiency shown in this work may be assessed as follows. In the cultures in which low calcium lengthened lag (L or L_g), an alternative metabolic pathway may have developed during this phase and this alternative pathway was not able to support as long an exponential phase as the normal pathway in

* This apparently happened in the work of Norris (1959) when many strains, after maintenance in synthetic medium, had their symbiotic characters altered.

cultures in which calcium was not limiting. The shortened exponential phase may perhaps be caused by increased excretion into the medium of toxic metabolites from calcium-deficient cells. This type of response to reduced calcium levels may also be explained by the deficient cells slowly accumulating a factor or factors at a rate determined by the calcium level of the medium: when a threshold value was attained, exponential growth started but was soon inhibited by the low calcium level. With the two strains whose growth rate in the exponential phase was reduced by low calcium, the effect may be attributed directly to the deficiency, since in this phase, growth is at a maximum for the conditions of temperature, aeration, and energy level provided.

Norris (1959) concluded that *Rhizobium* is not a calcium-sensitive organism and any requirement which it has for this element is of trace magnitude only. The work reported in this paper confirms this since quite good growth occurred with all strains with 0.1 p.p.m. calcium. However, the results also show that at this low level of Ca^{++} a deficiency was expressed in the form of the growth curves, in the total population attained and in a cell abnormality, when compared with growth in 10–14 p.p.m. calcium. For ordinary culture media, at least 10–14 p.p.m. calcium would be supplied as impurities in the constituents especially if agar is employed, but with purified materials, calcium should be added.

V. ACKNOWLEDGMENTS

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THE DIRECT ASSAY OF ^{14}C IN DRIED PLANT MATERIALS

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Summary

An apparatus is described and illustrated which enables the direct assay of ^{14}C in dried and ground plant material. The effect of five factors upon the measurement of the ^{14}C -content of the powder are discussed. It is concluded that this technique provides an efficient, accurate, and rapid method for the assay of ^{14}C in plant materials.

I. INTRODUCTION

More frequent use of ^{14}C -labelled materials is being made in plant physiology and many instances arise where one desires to assay rapidly and accurately the quantity of ^{14}C in a sample of plant tissue. In the past, this has been carried out by oxidizing the tissue and assaying carbon dioxide either directly as the gas or as barium carbonate (Calvin *et al.* 1949). Among the many methods of oxidation that are known, two have been used in our work: wet oxidation employing the modification of the Van Slyke method described by Calvin *et al.* (1949) and oxidation in oxygen as described by Schöniger (1960). Both these techniques do not deal adequately with quantities of dried organic matter in excess of 20 mg, and other procedures which can handle larger quantities are tedious. In studies of photosynthesis and the translocation of carbohydrates in plants, one often handles quantities of dried organic matter well in excess of 200 mg and to use the Van Slyke modification, it is necessary to mix and aliquot the tissue.

It was felt that the finely divided, dried, mixed powder of the plant tissues was itself suitable for measurement. Measurement of the ^{14}C -content of evaporated slurries of plant material has been reported by Zholkevich (1954), and MacKenzie and Dean (1950) have used briquettes of pressed plant material to measure ^{32}P . The activity of pure compounds has often been measured by solid counting (Calvin *et al.* 1949; Popjak 1950; Burr and Marcia 1955) and Popjak (1950) drew attention to the value of this technique as the activity of the material is not diluted during combustion to $^{14}\text{CO}_2$ and counting as $\text{Ba}^{14}\text{CO}_3$. However, no detailed discussion of the measurement of ^{14}C in plant tissues by the counting of dried powders has been reported.

II. EXPERIMENTAL

(a) Design

Five factors were tested for their effects upon the measured values of the radioactivity of the powder samples:

- A. The temperature at which the plant material was dried prior to grinding (60 and 110°C).
- B. The fineness of the grinding (to pass 30, 40, or 60 mesh).

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C. The relative humidity at which the powders were held prior to counting (5, 55, and 76%).

D. The thickness of the sample which was counted (10, 20, or 30 mg/cm²).

E. The position of the surface of the powder (level with the edge of the planchet or 1 mm below it).

TABLE 1

WEIGHT AND RADIOACTIVITY OF EACH SAMPLE ASSAYED

A = weight (mg) of sample counted. B = counts per 5-min period minus background (17.6 counts/min). L = surface height of powder 1 mm below shoulders of planchet. H = surface height of powder level with shoulders of planchet.

Plant No.		-1	-2	-3	-4	-5	-6	-7	-8	-9	Surface Position
1	A	60.2	40.0	20.4	60.2	40.5	60.4	40.3	20.9	20.8	H
	B	2638	2417	2689	2247	3185	3326	1968	1853	1957	
2	A	59.9	40.1	20.0	60.1	60.5	20.7	20.7	40.6	40.9	L
	B	986	968	694	815	851	808	814	808	818	
3	A	60.3	40.5	40.0	19.6	59.9	21.4	20.4	40.3	60.0	L
	B	1956	1322	1190	1225	405	1499	1723	562	1620	
4	A	20.5	40.6	59.4	40.7	66.4	19.6	40.3	60.2	20.6	H
	B	1880	2528	2476	2728	2353	2027	2418	2514	2069	
5	A	20.2	60.0	21.0	60.0	59.5	20.5	39.4	40.2	40.2	L
	B	1017	1093	1019	1255	1119	1001	1129	1166	1300	
6	A	20.5	40.5	60.5	20.7	39.0	60.6	60.4	40.4	20.8	H
	B	5082	5654	5488	4784	6349	4950	5560	5122	4234	
7	A	60.5	19.9	60.2	20.9	60.0	20.0	40.4	39.5	39.9	H
	B	2079	1747	2119	1970	2082	1710	2011	2019	2027	
8	A	19.8	40.1	60.1	40.5	60.3	19.4	40.5	56.6	20.4	L
	B	1385	1961	2264	2250	1945	2098	2599	1909	1744	
9	A	60.3	60.3	20.1	20.7	40.6	39.6	20.6	60.7	40.9	L
	B	1700	1625	1802	1481	1821	2003	1941	1988	1622	
10	A	60.9	19.9	40.7	19.5	59.3	39.9	40.9	60.5	20.1	L
	B	2762	2105	2883	2768	2636	2343	2579	2792	2421	
11	A	40.3	19.4	60.4	19.9	20.0	39.6	60.1	40.4	60.4	H
	B	2094	1597	1868	1925	1992	1854	2190	1865	1848	
12	A	40.7	19.6	20.5	40.0	40.5	59.5	20.1	60.5	60.4	H
	B	1885	1719	1263	1432	1778	1868	1510	1975	1846	

This was achieved by the use of a $2 \times 3 \times 3 \times 3 \times 2$ factorial experiment in which the effects of A, B, and E were measured by the differences between plants and the effects of C and D by the differences between subsamples of the same plant. The 12 plants necessary for the experiment were treated with ^{14}C -sucrose and dried, ground, and counted in a random order.

(b) *Method*

Twelve mature shoots of the tick bean (*Vicia faba* var. *minor*) each received 20 μg of ^{14}C -labelled sucrose, 10 μg on each of two leaves, applied with a micro-pipette. The plants were harvested, and dried for 3 days at either 60 or 110°C in an oven. They were then ground in a Wiley laboratory mill, Micro model, to pass either 30, 40, or 60 mesh. After grinding, the samples were mixed in revolving tubes overnight to ensure a thorough mixing. Each sample was subdivided into nine parts (labelled 1-1, 1-2, . . . , 1-9, 2-1, . . . , 12-9,) and the subsamples were placed for 5 days in one of three desiccators in which the relative humidity had been fixed at one of three levels (approx. 5, 55, or 76% R.H.). Nine subsamples from each of six of the plants were assayed on one day, and the nine from each of the other six plants on the next day. The radioactivity of each sample was measured for a 5-min period, and the results are set out in Table 1 showing the weight of the material counted, the counts per 5-min period, and the position of the surface during counting.

(c) *Apparatus*

A special apparatus was constructed to allow varying thicknesses of powder to be measured in a position of constant surface height. The apparatus is shown in Figure 1 and is composed of five parts:

- (1) A base which is mounted to the bench and carries a fixed locating ring to hold the planchets in a reproducible position.
- (2) A vertical spindle with two horizontal arms which can be rotated about the axis of the spindle. The head of the spindle carries a locking screw to lock the arms in position.
- (3) A Philips Geiger-Müller probe unit which is mounted vertically on one arm of the spindle and can be aligned accurately over the planchet for counting. The background count was 17.6 counts/min and therefore no further shielding was necessary.
- (4) A spring-loaded tool, accurately sized to the diameter of the space in the planchets, and made of light alloy to reduce the weight of the unit. The tip of the tool is made of cutlery grade stainless steel to lessen wear and corrosion.
- (5) The planchet: this was specially designed (Fig. 1(a)) so that the sample volume could be varied. The base of the area that holds the powder is carried on a screw and a hole through the base plate allows the surface of the plated material to be adjusted to a constant height.

To use this apparatus a sample of powder is placed in the weighed planchet which is then positioned in the ring on the base plate. The powder is tamped

gently with the plunger, and the surface adjusted by the screw in the centre of the planchet, retamped, and readjusted. This is continued until the surface is flat and level. Particles of the powder that inevitably occur on the edge of the planchet may then be removed gently with a brush. The plunger is swung out of position, the counter into position, centred, and the activity of the sample recorded. The weight of the sample and planchet is obtained and the mass of the sample calculated. The face of the plunger and the inside of the planchet are cleaned out and dusted and the unit is ready for a new sample.

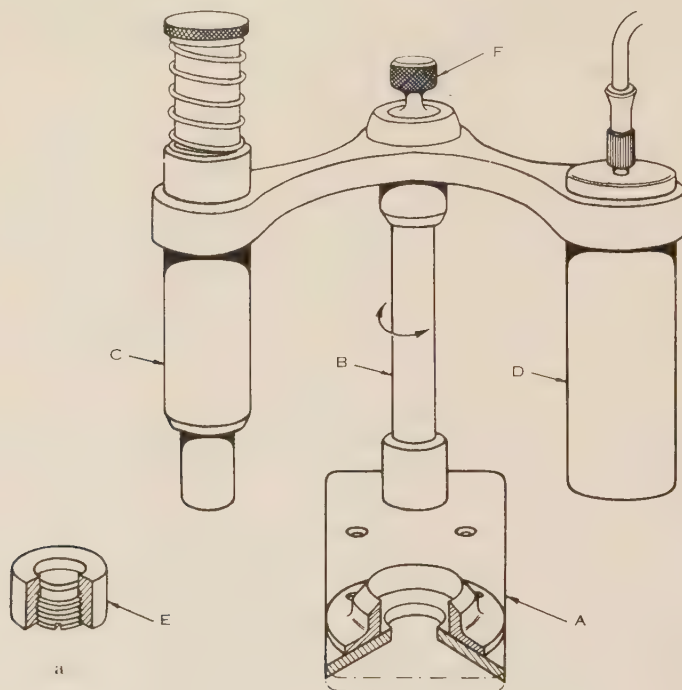


Fig. 1.—Details of apparatus used: *A*, base plate and locating ring; *B*, vertical spindle; *C*, spring-loaded plating tool; *D*, Philips probe unit; *E*, planchet; *F*, locking screw.

(d) Statistical Analysis

Since it was expected that the variability of a result would be approximately proportional to its magnitude, the analysis was carried out on the logarithm of the counts per 5 min. The Bainbridge tabular method (Bainbridge, Grant and Radoh 1956) was used to sort out the individual terms of the analysis of variance, a summary of which is set out in Table 2.

Four effects are seen to be significant:

(i) *The Drying Temperature*.—There is a highly significant increase in the counts per 5-min period when the material is dried at 110°C rather than at 60°C.

(ii) *Fineness of Grinding*.—The counts per 5-min period increase in a linear fashion with increasing fineness of grinding.

(iii) *Surface Height*.—There is a highly significant effect upon the count rate if the geometry is altered by changing the position of the surface by 1 mm: the counts are lowered when the surface is 1 mm further away from the counter tube.

TABLE 2
ANALYSIS OF VARIANCE

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Significance
<i>Between plants:</i>				
Drying temperature (A)	1	1.913605	1.913605	**
Fineness of grinding (B)				
B ₁ linear effects	1	0.348612	0.348612	*
B ₂ quadratic effects	1	0.159522	0.159522	N.S.†
A × B ₁ interaction	1	0.212986	0.212986	N.S.
Surface height (E)	1	1.213488	1.213488	**
A × E interaction	1	0.047544	0.047544	N.S.
B ₁ × E interaction	1	0.074369	0.074369	N.S.
Error	4	0.170859	0.042715	
<i>Within plants:</i>				
Conditioning humidity (C)				
C ₁ linear effects	1	0.013778	0.013778	N.S.
C ₂ quadratic effects	1	0.002373	0.002373	N.S.
Deposit thickness (D)				
D ₁ linear effects	1	0.037766	0.037766	*
D ₂ quadratic effects	1	0.007573	0.007573	N.S.
Two-factor interactions	20	0.134578	0.006729	N.S.
Error				
Three-factor interactions	36	0.238644	0.006629	
Four- and five-factor interactions	36	0.266023	0.007389	
Pooled errors	72	0.504667	0.007009	
Total	107	4.712578		

* Significant at 5% level.

** Significant at 1% level.

† N.S., not significant.

(iv) *Thickness of Deposit*.—There is a linear increase in the counts per 5-min period as the weight of sample counted increases from 10 mg/cm² to 30 mg/cm².

The fifth effect, the level of conditioning humidity, did not affect the count rate significantly.

III. DISCUSSION

(a) *Drying Temperature*

As mentioned above, the count rate of samples dried at 110°C is higher than that of samples dried at 60°C. It has not been possible to attribute this effect to any one simple factor. The moisture retained by the tissues, their densities, and

the thickness of 30 mg/cm² are identical. Since the moisture content and density of the two powders are constant and the geometry is not varying one is forced to conclude that the effect observed is probably due to some property of the surface of the powder. While it is disappointing to be unable to attribute the effect to any specific factor, it is of no practical importance to users of the technique as one ordinarily dries plants at one particular temperature. Further, since several authors have commented on the loss of ¹⁴CO₂ from tissues during high temperature drying, vacuum drying at reduced temperatures or "freeze drying" are to be preferred (Calvin *et al.* 1949; Pirie 1956).

(b) *Fineness of Grinding*

This effect was small, and considered alone, there would be no point in grinding samples to pass finer than 30 mesh. The small increase in count rate of the sample would not be worth the extra time required to grind the sample to pass finer meshes.

However, the variability of the counts of samples ground to pass 30 mesh is very significantly higher than that of samples ground to pass 60 mesh, the coefficients of variation being 34 and 10% respectively.

The coefficient of variation for a result derived in the above experiment is 20%. Although this in no way invalidates the experiment, this is large and led to doubts about the accuracy of the technique. Several tests were carried out subsequently to this experiment in which samples of powder were ground to pass 40 mesh, mixed overnight by rolling in a tube, and plated at 30 mg/cm² at constant surface height. Under these conditions the technique yielded results with a coefficient of variation of less than 3%, which in most cases is superior to the precision of results obtained when tissues are oxidized and counted as Ba¹⁴CO₃. The high variability in the former experiment arose because the subsamples were poorly mixed before they were counted.

(c) *Level of Conditioning Humidity*

The technique is rendered more useful since this factor does not significantly affect the count rate of the samples. Samples can therefore be ground, mixed, and counted without equilibration.

(d) *Deposit Thickness and Surface Height*

Plant powders prepared by the technique described above have a large volume/mass ratio. If, as was done here, the height of the surface of the sample is kept constant at different thicknesses by the use of the screw levelling device described, then the count rate of samples of different thicknesses may be corrected for self-absorption losses by the standard methods (Calvin *et al.* 1949). If this levelling device cannot be incorporated, increasing thicknesses of powder plated in standard planchets do not display an "infinite thickness" value, the count rate of the sample rising continually with increasing thickness. This is caused by the change in geometry of the counting system brought about by plating increasing thicknesses of material.

Three ways of overcoming this difficulty are suggested:

- (i) A constant thickness of material may be used—this becomes tedious when many samples are to be counted.
- (ii) One may work with thicknesses where the count rate is a linear function of thickness.
- (iii) A self-absorption correction curve may be prepared to suit the particular circumstances.

It is important to note that coefficients of variation of less than 3% have been obtained using the last technique in conjunction with standard planchets, provided suitable thickness corrections are made. It is also reassuring to note that no two-factor interactions were significant. Thus the differences in count rate produced by alterations in one variable do not themselves vary significantly with changes in any other of the variables that were tested.

It is often necessary to compare the radioactive concentrations of various plant organs relative to one another and it is important that the ratios of activities be independent of the assay technique.

The relative activities of various organs were compared by the direct assay of the powdered material and by the normal method following oxidation, and the ratios were found to agree within the limits of experimental error (approx. 5%).

The simplicity of the operations, the cheapness of apparatus, and the speed with which large numbers of plants may be assayed are all pointers towards widespread use of this technique. Other methods of grinding should also be applicable and there seems to be no theoretical reason for limiting its use to assay of tracers in plant material: it may be possible to assay desiccated faeces, blood, or animal tissues if these materials yield powders with satisfactory properties.

IV. ACKNOWLEDGMENTS

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THE FREEZING OF PLANT TISSUE

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Summary

A theory of the freezing process of the tissue of pear fruit is presented, based on the cell permeability theory and on the thermodynamics of moisture in porous media. According to this theory the nature of the temperature *v.* time curve during freezing is determined by the speed of advance of the ice front into the tissue. Curves for freezing and thawing calculated from the theory are compared with experimental curves.

Using the theory to interpret the experimental freezing curves, further experiments enabled the true freezing point of the tissue to be deduced. In most cases the true freezing point, defined as the highest temperature at which ice can propagate through the tissue, coincided with the freezing point of the expressed juice. In some cases, however, the true freezing point was as much as 1°F below the juice freezing point.

The theory provides an explanation for a number of points raised in the literature which are either unexplained or the subject of dispute.

I. INTRODUCTION

As part of an investigation into the freezing point of pear fruit, a detailed study has been made of the temperature *v.* time curves followed by intact pears and pieces of their tissue during freezing and thawing. The practical purpose of this work was to examine the relationship between the maximum freezing temperature exhibited by the freezing curves (Fig. 1) and the true freezing point, which is defined as the highest temperature at which ice may form throughout the tissue. A theory is put forward, based on the cell permeability theory and on the thermodynamics of moisture in porous media, from which freezing and thawing curves can be calculated and compared with the experimental curves. This theory is presented here together with a discussion of its bearing on current ideas on the freezing of plant tissue—an account of the more practical implications of this work, and further investigations of the practical problem will be published elsewhere.

II. EXPERIMENTAL

Temperatures were measured with a recording thermistor thermometer developed by Melville (1958), which uses a probe consisting of a 20-gauge hypodermic needle with a thermistor bead set in the tip. The output is recorded on a 1-mA Esterline Angus recorder. The instrument was adjusted and calibrated against a standard thermometer for the range 22–32°F, but temperatures beyond this range could be read by reducing the sensitivity in known ratios.

The pears were frozen in the modified freezing chamber of a domestic refrigerator. A plywood lining opening at the top was fitted, leaving a 1-cm air gap between

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it and the metal evaporator. This in conjunction with a Danfoss thermostat enabled the air temperature to be controlled within a range of 0.4°F .

For thawing experiments a double-walled container with ice-water in the outer jacket was used.

In preliminary experiments many freezing curves were recorded, typical examples of which are shown in Figures 1 and 2. Features of the curves for intact pears are that there is always some supercooling, quite often as much as 10°F (Fig. 1, *B*); when freezing does begin it may take as long as 1 hr for the temperature to rise to its maximum; after this the temperature falls steadily, the rate of fall increasing gradually, or sometimes fairly suddenly after 6 or 8 hr as in Figure 1, *A*. A conspicuous feature in every case is a sharp pulse which very often rises above the maximum freezing temperature, and which may occur anywhere during the

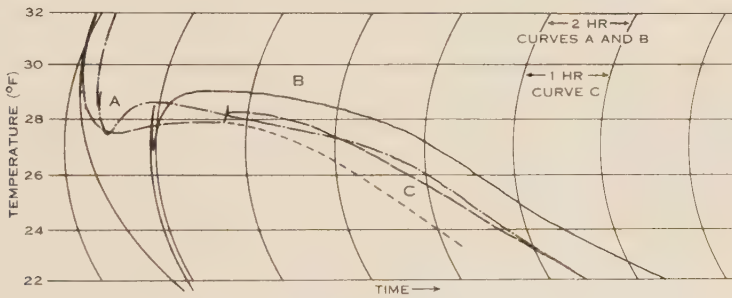


Fig. 1.—Experimental records showing freezing curves for intact pears, placed in an environment at 15°F . (The dashed curve in *C* is an addition to the record which is referred to later in the text.)

supercooling stage, although very occasionally it may occur as late as in Figure 1, *C*. If the pear is dissected when the curve is at the maximum, it is found that only a comparatively thin layer of tissue just under the skin is frozen—about 0.5 cm thick or less. If the pear is not dissected until the temperature has fallen 1 or 2°F below the maximum, there will usually still be found a small region of unfrozen tissue. The presence of freezing is shown by the translucent appearance of the tissue, but if the pear has been frozen in an environment colder than about 20°F the distinction of the frozen from the normal, white, opaque, unfrozen tissue is not very clear immediately after dissection. After 15 – 30 min at room temperature, however, a sharp division between the two regions (which one may call the “ice front”) is apparent. The thickness of the frozen layer is usually quite uniform, although in some cases it has a spotty appearance, the centre of some of the spots coinciding with the vascular bundles. The frozen region also extends around the thermistor probe, in a cylinder of radius comparable with the thickness of the surface layer.

If an intact pear is placed in a comparatively warm environment, at say 25°F , it is not practicable to obtain a freezing curve at all, as the pear will remain supercooled at this temperature for 24 hr or more. If the fruit is peeled first, however, a curve such as that in Figure 2, *A* is obtained. The characteristics of such curves

are only slight supercooling, a sudden temperature rise of about 1°F , followed by a temperature fall which settles down after about an hour to a very steady rate. In this case when the tissue is dissected the translucent frozen region is immediately distinguishable from the unfrozen tissue.

When peeled fruit or pieces of tissue are frozen at lower temperatures, the main difference from the curves of Figure 1 is that deep supercooling never occurs. In some cases in fact curves like Figure 2, *C* are obtained, in which, apart from the sharp pulse, the curve never becomes horizontal.

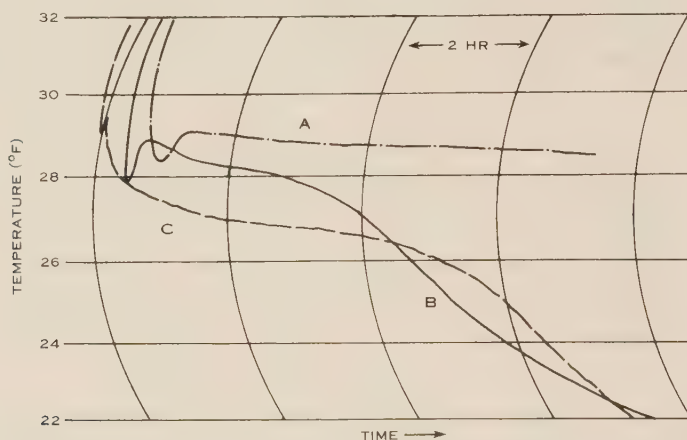


Fig. 2.—Experimental records showing freezing curves for peeled fruit and pieces of tissue. The environment temperatures were: A, 24°F , B, 19°F , and C, 17°F .

These experiments showed that the freezing temperatures were dependent on the temperature of the environment, the recorded curve deviating from its general trend to follow variations in the environment temperature. The variations in the temperature of the tissue, however, were only about 5% of those of the air in the freezing chamber.

To provide a temperature characteristic of the tissue, against which freezing temperatures could be compared, the freezing point of the juice was measured. The "juice freezing point" is here defined to be the measurement obtained by freezing tissue hard overnight (at 15°F or lower), thawing it, and then refreezing it, preferably at the same low temperature. A piece about 1 by 1 by 2 cm freshly cut is used for the second freeze. With a piece this size or larger, the resulting freezing curve has a reasonably flat top which is repeatable and can be measured to 0.1°F accuracy. It was confirmed that this value is identical with the freezing point obtained for the expressed juice. This refreezing method is very simple to use for routine measurements, and will measure the juice from a restricted locality.

The maximum freezing temperature of intact fruit held at fairly low temperatures may be about 2°F below the juice freezing point—a well-known result. While this freezing temperature was evidently not the highest temperature at which

the tissue could freeze, it was a principal aim of the investigation to determine whether the true freezing point was as high as the juice freezing point.

III. PREVIOUS WORK

There is a vast literature dealing with the freezing of plant tissue which has been extensively reviewed by Levitt (1941, 1958). This literature is mostly concerned with the nature of frost injury. The present investigation, however, was limited to determining the highest temperature at which freezing can occur—whether or not the freezing was injurious was not considered.

Levitt concludes that the evidence supports the permeability theory. Briefly this theory states that at comparatively mild freezing temperatures ice formation is confined to the intercellular spaces, supercooled water from inside the cell diffusing through the cell wall to freeze on the ice outside. Only when the temperature falls below a certain limit does intracellular freezing occur.

The various modes of ice formation are shown very graphically in the cinemicrographs of Modlibowska and Rogers (1955) which the author was fortunate enough to see. The growth of ice by withdrawal of water through cell wall or membrane could be observed under various circumstances. Of particular interest was the progress of intracellular freezing along a staminal hair of *Tradescantia*. The wall between successive cells was stretched sometimes to four or five times its original size before ice crystals were able to pass through it, after which the cell contents froze very quickly and the process was repeated at the next intercellular barrier. The same process is described in detail by Asahina (1956).

A physical explanation of why ice crystals may not pass through the porous cell wall to the supercooled water on the other side has been given by Jackson and Chalmers (1958). Their work is concerned with soil moisture but is equally applicable to plant tissue. They show that the interfacial free energy of the ice-water interface has the effect of lowering the freezing point of water in a capillary by an amount inversely proportional to the radius of the capillary (provided the other interfacial free energies obey a certain condition). One can see that if the cell wall is stretched, enlarging the pores, a stage will be reached at which the water in the pores can freeze, and the ice crystals grow into the supercooled liquid beyond.

Concerning actual freezing temperatures, the literature is not conclusive. Levitt (1941) remarks "Many recent workers have confirmed the claim that dead tissues freeze at a higher temperature than living tissues. The lower the temperature of the freezing bath the lower was the freezing point of the tissue." And again, "Thus, though the freezing point of living tissue varies with the conditions, it has not been possible to obtain real agreement with the freezing point of the same tissue when dead." Maximov (1914) was evidently the first to point out that a consequence of the permeability theory is that the freezing temperature of plant tissue must be below that of the free cell sap.* Thus (as quoted by Levitt) "The freezing point of living tissue depends on a complicated interaction between the rate of cooling,

* I am indebted to the late E. W. Hicks, Division of Food Preservation, C.S.I.R.O., for first drawing my attention to Maximov's work and the permeability theory.

the resistance of the protoplasm to the exit of water, and the speed of ice formation in the intercellular spaces." ". . ., there must be a steady drop (in temperature), slow at first, then more rapid, due to the progressively smaller quantity of ice formed and of heat liberated thereby." E. W. Hicks (personal communication) had suggested that the true freezing point of living tissue should not be more than 0.1°C below the freezing point of disrupted tissue.

Our preliminary observations were generally consistent with the permeability theory. The appearance of the translucence on dissection, for instance, is in agreement with the occurrence of extracellular ice formation during mild freezing and intracellular ice formation during more rapid freezing. The translucence is evidently caused by the replacement of the air in the intercellular spaces by water, and the consequent removal of the air-water interfaces round each cell and the refraction of light which they produce. In the case of the more severe freezing, it takes some minutes for the melting ice inside the cell to seep through the walls into the spaces, whereas after mild freezing the ice is already in the spaces and translucence appears immediately.

To explain the details of the freezing curves, however, a more precise formulation of the theory is needed. The very existence of the ice front shows that the cells do not all freeze at the same temperature in a given case, and the presence of unfrozen tissue when the temperature has fallen 2°F below the minimum freezing temperature shows that either some mechanism comes into play which progressively lowers the freezing point, or that supercooling of the tissue may be very persistent. In the latter case Levitt's suggestion that intracellular ice results when "the temperature of the cell sap falls below its undercooling point" cannot hold. A full understanding of the freezing curve must take into account the flow of heat through the pear tissue.

IV. THEORETICAL

The theory proposed here is an elaboration and quantification of the permeability theory. It asserts that the shape of the freezing curve is determined by the speed of advance of the ice front, or more fundamentally, by the factors which determine this speed. In the case of a single body of liquid the speed of the solidification front is determined by the balance between the rate of heat loss from the front and the rate of latent heat release. This balance occurs when the temperature remains steady at the freezing point of the liquid—the temperature at which the specific free energies of the liquid and the solid are equal. If the speed were to decrease, the balance would be disturbed in the direction which would make the temperature fall, which would result in the liquid having a greater free energy than the solid. This energy difference would cause a force tending to restore the balance.

In a cellular tissue, however, the speed of the ice front is dependent on the rate at which water diffuses through the cell wall, which in turn depends on the degree of supercooling (since this provides the driving force). It is an essential postulate of the present theory that once freezing has begun the remaining intact cells do not freeze until they come within the influence of the ice front.* During

* Both Modlibowska and Rogers (1955) and Asahina (1956) emphasize the stability of cells under supercooling.

extracellular freezing a layer of ice is visualized as growing over the cell wall, regularly bringing new cells within its water-extracting influence as it comes in contact with them. When the freezing is intracellular it is visualized as being transferred from cell to cell in the manner filmed by Modlibowska and Rogers. In this case not only must water diffuse through the cell wall, but the ice formed must stretch the wall until the pores are sufficiently enlarged for the water in them to be able to freeze. It can be seen that more energy will be needed for this process than for the straightforward diffusion in extracellular freezing, and therefore the temperature must be lower. This explains why even if a cell should freeze internally, it will not cause adjacent cells to freeze in a similar manner unless they are supercooled beyond a certain limit. This is discussed in more detail below.

It will now be shown that by assuming a suitable relation between the speed of the ice front and its temperature, freezing curves can be calculated which have the same general characteristics as the experimental curves shown in Figures 1 and 2. These theoretical curves enable meaningful comparisons to be made between experimental curves of different shapes, which in turn give an indication of the true freezing point of the tissue.

Calculation of the Freezing Curve

In applying mathematical methods to phenomena in biological material which displays so much variety that no two samples are ever identical, it is necessary to make numerous generalizations and simplifications. This approach has been ably expounded and justified by Rashevsky (1948) in the preface of his book "Mathematical Biophysics". In the present case we begin by assuming that the pear or piece thereof is spherical and that the ice front lies on a concentric sphere. We will be satisfied if the values of the physical constants used are only approximately correct.

The temperature of the environment is taken as zero and the following temperatures defined (in Centigrade degrees):

V_0 = the ice point,

V = the true freezing point of the pear tissue,

v_F = the temperature of the ice front (a function of time),

v = the temperature of any point in the pear (a function of radius and time).

Also let

a = radius of the pear (in cm),

ρ = radius of the ice front (in cm, a function of time).

At this stage we make the arbitrary assumption that:

$$\text{Speed of ice front} = -d\rho/dt = S(V_0 - v_F) \text{ cm/sec for } v_F < V, \quad (1)$$

where S is a constant. The reasons for this assumption will be discussed later. In the meantime it may be remarked that the speed increases with the supercooling as desired.

When a cell at the ice front freezes, whether externally or internally, it will be assumed that it takes negligible time for all the water which can freeze at the temperature v_F to do so. It then follows (see Appendix I) that the latent heat l

(cal/g) released is given by:

$$l = L[(V - v_F)/(V_0 - v_F)], \quad (2)$$

where L = latent heat when all the water is frozen. This expression implicitly assumes that the effect of anything other than dissolved material which causes a depression of freezing point remains constant for all values of the temperature v_F .

After the ice front has reached a particular cell and it has become a part of the frozen region between the surface of the pear and the ice front, it will undergo thawing or further freezing according as the local temperature rises above or falls below the temperature at which it originally froze. If it is assumed that the freezing point of the unfrozen solution takes negligible time to adjust itself to the local temperature, the effect of this marginal freezing and thawing is equivalent to a temperature-dependent specific heat of magnitude:

$$-dl/dv = L(V_0 - V)/(V_0 - v)^2 \text{ cal/g } ^\circ\text{C}. \quad (3)$$

The shape of the cooling curve before freezing begins shows that it is a good approximation to assume that the heat loss from the surface of the pear is proportional to the difference between the temperatures of the surface and the environment—sometimes called the “radiation” boundary condition (Carslaw and Jaeger 1948, pp. 13–16):

$$\text{Rate of heat loss} = H v_s \text{ cal/cm}^2 \text{ sec}, \quad (4)$$

where v_s is the temperature of the surface (the temperature of the environment being defined as zero), and H is a constant.

In common with most heat flow problems involving latent heat, the present one cannot be solved analytically. The alternative method is to use numerical analysis, the usual method for a partial differential equation like the heat equation being to use a space lattice of points and calculate the temperature distribution at successive time intervals. This method was not used here, however, as without an electronic computer the labour involved is usually prohibitive.

A simpler though somewhat less accurate method is to consider the heat balance of the pear as a whole:

(Rate of heat loss at surface) — (rate of latent heat release)

= Net rate of heat loss from pear

= (Volume) \times (density) \times (average specific heat) \times (rate of temperature drop). (5)

This approach leads to an ordinary differential equation which can be solved more easily. The details are given in Appendix I.

A family of solutions to the equation is shown in Figure 3. A family rather than a single curve is obtained because the temperature at which freezing begins can be chosen arbitrarily. In curve *A* freezing begins when the temperature has fallen 0.9°F below the true freezing point of the tissue, V . Nevertheless, the temperature continues to fall quite rapidly for another $1\frac{1}{2}^\circ\text{F}$. Note the resemblance between this curve and the experimental one in Figure 2, *C*.

In curve *D* the tissue was considerably supercooled before freezing was assumed to begin. The temperature immediately rose to a rounded maximum similar to the majority of the experimental records for intact fruit (Fig. 1). Curve *B* may be

regarded as the most fundamental one from a theoretical point of view. In this case the rate of heat loss and the rate of heat release are equal at the instant when freezing begins. The temperature at the point *B* will be defined as the "initial freezing temperature".

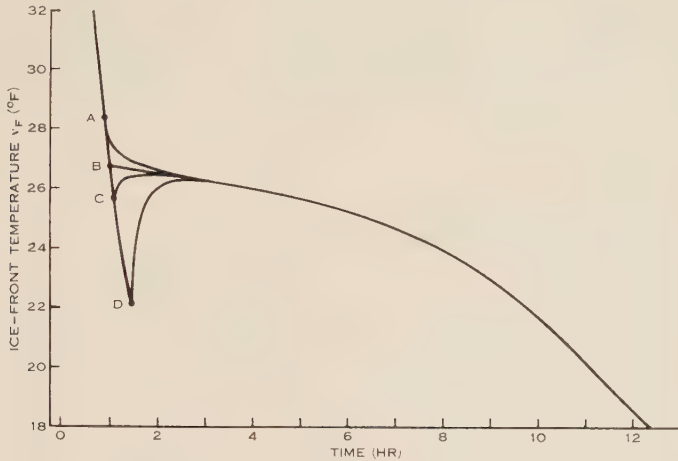


Fig. 3.—Theoretical curves for the temperature of the ice front in a pear of radius 3 cm having a true freezing point of 29.3°F , frozen in an environment at 14°F . The ice-front speed factor, S , was taken as 2×10^{-6} cm/sec $^{\circ}\text{C}$. The values of other constants used are given in Appendix I.

One of the points of detail in which the experimental records differ from the theoretical curves in Figure 3 is that the bottom of the supercooling minimum may be gently rounded. Part of the explanation for this lies in the time required for the

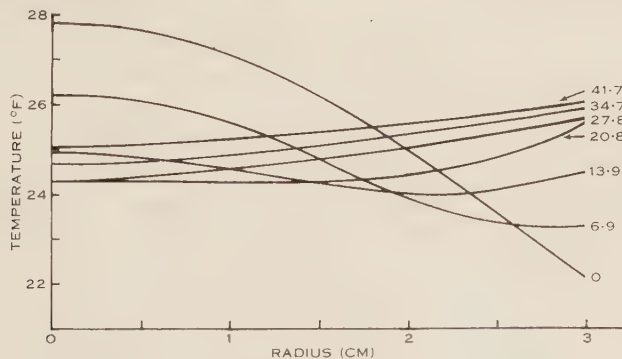


Fig. 4.—Theoretical temperature distributions within the pear at certain times after the instant represented by point *D* in Figure 3. Times (in minutes) are indicated on the figure.

latent heat released from the ice front at the surface to penetrate to the centre of the fruit. The temperature distribution within the pear at successive times for the case *D* of Figure 3 is shown in Figure 4. The temperature at the core continues to

fall for 21 min after it has begun to rise at the surface. The variation with time of the temperature at radius 1 cm may be compared with the temperature of the ice front in Figure 5. The "dashed" part of the curve is an estimate based on the fact that the interior temperatures follow that of the ice front closely when the temperatures are not changing rapidly. At the point *P* the ice front is at radius 1 cm and the curves coincide. At later times the points at 1 cm are in the frozen region and the temperature falls below that of the ice front. The curve of ice-front temperatures is thus the pattern from which the temperature at any particular point differs in a predictable way.

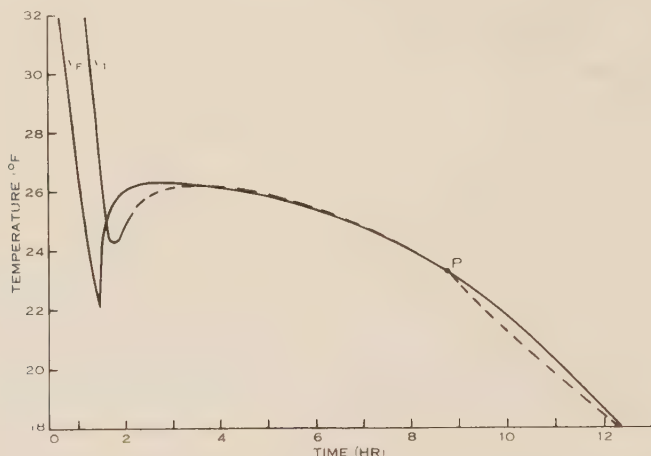


Fig. 5.—Theoretical curves for the ice front temperature, v_F , and the temperature at radius 1.0 cm, v_1 , for case *D* of Figure 3.

Another reason for the rounded minimum is that once freezing has begun at a comparatively few (or perhaps only one) points at the surface, it takes a certain length of time for the freezing to spread and form a continuous ice front. Since the supercooling is deepest at the surface and rapidly increases at areas which have not frozen, the ice front tends to grow round the surface initially rather than into the warmer interior. It is only when the ice front is almost complete that the latent heat begins to raise the temperature of the interior. In the case of peeled fruit or pieces of tissue the surface is covered with a layer of juice from the damaged cells, which may be expected to freeze throughout very quickly. As would be expected, deep supercooling does not often occur in these cases and when it does the temperature rises rapidly once freezing begins. A further effect which will be discussed in more detail below is due to the freezing of the tissue immediately surrounding the probe.

With the use of only a desk calculator the number of solutions of the differential equation had to be restricted to a few typical cases. It would be highly desirable to run this problem on an electronic computer and to observe the effect of varying the constants. One variation which has been calculated is to double the value of the speed factor S (see Fig. 6). The general shape of the curve has not changed, but as the ice front travels from the surface to the centre its temperature falls by only 4.2°F instead of 6.8°F as in the first case.

Another case which has not been treated concerns the freezing point of the tissue, V , which has been taken as constant throughout the volume. In practice, however, one finds that the juice freezing point is almost always higher at the core than just under the skin, the difference frequently being as much as 0.8°F . Since the freezing point of the tissue must be closely related to, if not identical with, the juice freezing point, it would be desirable to know the theoretical effect of making V decrease with the radius. Presumably the temperature would not fall quite so rapidly from the maximum freezing temperature.

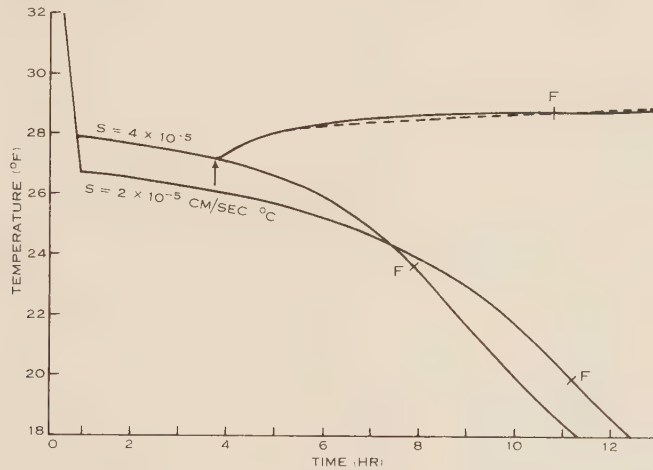


Fig. 6.—Theoretical curves showing the effect of doubling the value of the speed factor, S ; and also the thawing curve when the pear is transferred to an environment at 32°F at the moment when the ice front is at radius 2.0 cm . The points marked F show the moment at which the ice front reaches the centre; the full lines represent the ice-front temperature (or the temperature at the centre after the points F). The dashed line represents the temperature at radius 1.5 cm during the thaw.

Although the full possibilities of this theory have not been explored in detail, it is clear that it can account for the main features of the experimental records. The basic physical meaning of the freezing curve is that the rate at which the temperature changes is determined by the difference between the heat lost and the heat released. The heat loss falls quite slowly, since the area of the surface of course remains constant while the rate of heat loss falls with the surface temperature. On the other hand the latent heat released falls quite rapidly as the ice front moves in towards the core, since the decrease in area with the square of the radius predominates over the increase of speed of the ice front with falling temperature. The exact rate of temperature change which results from a particular heat loss or gain depends on the average equivalent specific heat of the pear. At first this specific heat rises as the frozen region increases in volume, but eventually it decreases again as the specific heat due to marginal freezing (eqn. (3)) decreases with falling temperature. This means that for a constant net heat loss the rate of temperature drop would gradually decrease at first and then speed up again.

One of the more puzzling experimental observations was the presence of unfrozen tissue when the temperature had fallen well below the maximum freezing temperature. On the present theory this is not to be attributed to any mechanism which causes an actual depression of freezing point of the tissue, but to the high stability of the cell sap in the supercooled state. A particular cell can be made to freeze only by inoculation with ice crystals from an adjacent cell. Confirmation of this view is provided by a study of the thawing process treated below.

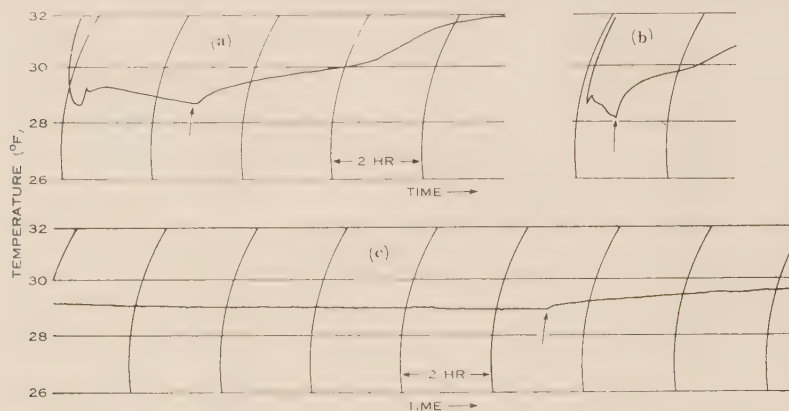


Fig. 7.—Experimental records showing thawing curves. The temperatures of the freezing environment were: (a) 26°F, (b) 11°F, and (c) 26°F. The arrows indicate when the pieces of tissue were transferred to the thawing chamber (32°F).

It will be noticed that according to this theory the maximum freezing temperature has no direct relation to the true freezing temperature of the tissue, V . It still had to be determined, however, whether V was identical with the juice freezing point. Some light could be thrown on this question by studying the relation between the initial freezing temperature and the temperature of the freezing environment, which is considered below. The theoretical curves in Figure 3 are of great value here in enabling the initial freezing temperature to be estimated even when freezing did not begin at the point B .

V. THAWING CURVES

If during the freezing process the pear is suddenly transferred to an environment held at 32°F, the temperature follows a course of which typical examples are shown in Figure 7. The features of the thawing curve are a fairly sharp rise in temperature of 0.2–1.2°F, which begins within about $1\frac{1}{2}$ min of the transfer to the thawing chamber; a slow but very steady temperature rise for some hours; followed by a gradual increase in the rate of warming.

The most striking feature of the curves is the sudden initial temperature rise. A brief calculation shows that this cannot be caused by the heat flowing in from the surface; $1\frac{1}{2}$ min is in fact the order of time required for the surface heat to just begin to reduce the temperature gradient which causes heat to flow outwards from the ice front. It would take much longer than this for the gradient to be reversed and

cause heat to flow inwards. The explanation of the effect lies in the fact that, although a thawing process has begun at the surface of the pear, the ice front continues to advance as long as the cells just inside the front are supercooled below their freezing point, V . Rather paradoxically, it is in fact the latent heat of freezing at the ice front which causes the initial temperature rise of the thawing curve.

A theoretical thawing curve has been calculated for one case (Fig. 6). Since there was no alternative to the laborious space lattice method of calculation, only a portion of the curve was calculated. The temperature distribution at various instants is shown in Figure 8. It can be seen that in this particular case the ice front

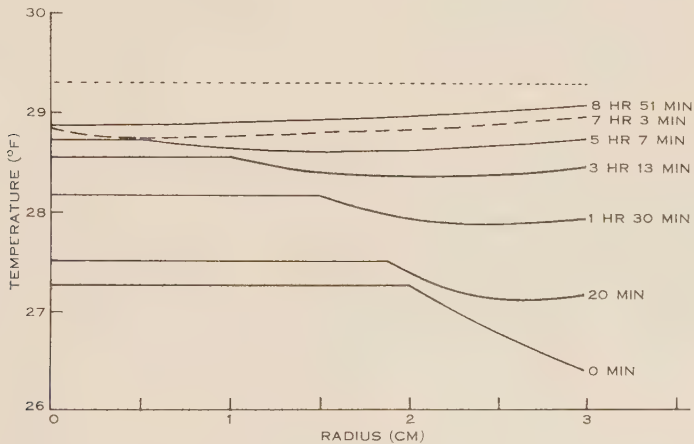


Fig. 8.—Theoretical temperature distributions within the pear at certain times (indicated on the figure) after the moment of transfer to the thawing environment at 32°F. Although the temperature inside the ice front was assumed to be constant as shown here, theory shows that the temperature at the centre would differ from that of the ice front by 0.1°F at the early stages of thawing. The dotted line shows the true freezing point, $V = 29.3^{\circ}\text{F}$.

continues travelling till it reaches the centre although thawing began when the front had reached a depth of only a third of the radius of the pear. (If thawing had begun earlier the temperature would have risen faster, and the ice-front temperature risen to the freezing point V before the front reached the centre.) For the first 7 hr of thawing both freezing and thawing are proceeding simultaneously. The history of any cell near the centre is that as the ice front reaches it, it suddenly freezes. Then as the front passes on it slowly thaws, the latent heat of melting at first coming from the ice front but later coming from the outside.

This interpretation of the thawing curves was confirmed by two experiments. In the first, two pieces of tissue cut from the same fruit were frozen for $1\frac{1}{2}$ hr at 11°F . Then, as the one whose temperature was being recorded was transferred to the thawing chamber, the thickness of the frozen layer in the other was measured. When the first piece was examined after thawing for 3 hr it was found that the thickness of the frozen layer had approximately doubled, confirming that the ice front continued to advance during thawing.

In the second experiment a piece of tissue was frozen overnight at 12°F and then left to reach equilibrium in an environment at 26°F for 24 hr. This ensured that it was evenly frozen throughout, with no ice front. When this piece was transferred to the thawing chamber, there was no sudden temperature rise; in fact, it took from 10 to 15 min for the usual slow temperature rise to become established.

VI. FREEZING ROUND THE PROBE

A conspicuous feature of every experimental record which has not yet been referred to in the theoretical treatment is the sharp pulse which usually occurs at an early stage of the freezing. All the evidence indicates that this pulse is caused by freezing of the ruptured cells at the probe itself, as suggested by Maximov. The very sharpness of the pulse is strong evidence for this view. A heat pulse which originated at a distance from the probe would quickly become diffused in the process of conduction to it.

After the effect of the sharp pulse has disappeared, the expanding ice front round the probe will raise its temperature above that of the surrounding tissue. An approximate idea of the magnitude of this effect can be obtained by considering the temperature of a point from which an ice front expands radially outwards. It is found that if this is the only freezing taking place, the latent heat will not be sufficient to raise the temperature of the point but will simply slow down the rate at which the temperature falls. As suggested above, this is probably one reason for the rounded minimum in freezing curves such as Figure 1, *A*.

If, on the other hand, the frozen shell which grows inward from the surface of the pear has formed, the effect of the freezing round the probe is to lift the temperature of the probe some fraction of a degree above the temperature of the outer ice front. The magnitude of the difference which occurs in practice can be obtained from the few curves like Figure 1, *C* which were recorded. It can be seen that the curve has been displaced upward by about 0.3°F following the sharp pulse. Since calculation shows that this difference must gradually increase, the record which would have been obtained in the absence of freezing round the probe is probably as shown by the dashed line.

The practical importance of this question is that all those records—the vast majority—in which the pulse occurred at an early stage, are displaced upward compared with the theoretical curves. This has been ignored, however, in the experiments described in the next section, since any error will be on the safe side.

In the case of peeled tissue frozen gently (Fig. 2, *A*), the pulse may be quite broad. It is likely that this is due to the infiltration of juice from the ruptured cells into the spaces between the adjacent layers of cells, which increases the speed of the ice front through these layers. The broad pulse has accordingly been ignored in the extrapolation.

VII. RELATION BETWEEN INITIAL FREEZING TEMPERATURE AND ENVIRONMENT TEMPERATURE

The initial freezing temperature has been defined as the temperature at which the rates of heat loss and latent heat release are equal at the instant freezing begins.

An expression for this temperature can be obtained from equation (8) in Appendix I, by equating dv_F/dt to zero with $\rho = a$. This gives:

$$LS(V-v_i) = H v_i, \quad (6)$$

since both v_F and v_s now equal the initial freezing temperature, v_i .

Reverting now to the normal centigrade system with the ice point taken as 0° , we define:

T_0 = temperature of the environment ($= -V_0$),

T = true freezing point of tissue ($= V + T_0$), and

T_i = initial freezing temperature ($= v_i + T_0$).

Equation (6) then becomes:

$$LS(T - T_i) = H(T_i - T_0)$$

or

$$T_i = [H/(H + LS)]T_0 + [LS/(H + LS)]T. \quad (7)$$

Since H , L , and S are constants for a particular pear, this means that if a pear is cut into a number of pieces which are frozen separately at different environment temperatures, the plot of initial freezing temperatures against environment temperatures should give a straight line which intersects the line $T_i = T_0$ at the point $T = T_i = T_0$, i.e. at the true freezing point.

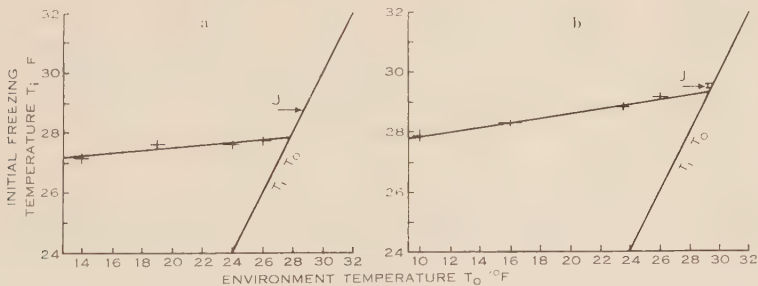


Fig. 9.—Experimental results for two pears, both Winter Nelis. In case (a), which was classed as “reasonably good”, only one juice freezing point measurement, J , was made. In case (b), classed as “very good”, the core and the surface juice freezing points differed by only 0.2°F .

Such an experiment has been carried out on 18 individual pears, typical results being shown in Figure 9. Agreement with the theory has been assessed as very good, reasonably good, or inconclusive according as values of the freezing point T resulting from all straight lines which reasonably fit a set of points, lie within the respective ranges: less than $\pm 0.15^\circ\text{F}$, between ± 0.15 and $\pm 0.25^\circ\text{F}$, and greater than $\pm 0.25^\circ\text{F}$. On this basis 8 results could be classed as very good, 5 as reasonably good, and 5 as inconclusive. The aim of these experiments was to relate the true freezing point T to the juice freezing point. The results for those pears which fell in the first two categories are given in Table 1.

Since the practical purpose of this investigation was to establish the lowest temperature at which a pear could be stored without any danger of freezing, the values in the table have been chosen where necessary, on the safe side. When the juice freezing point, for instance, was higher at the centre than at the surface of the pear, the lowest value has been used. Similarly the initial freezing temperatures were obtained by direct extrapolation from the experimental curve, as the point *B* in Figure 3. The effect of freezing round the probe is probably to make the value so obtained too high if anything.

The practical conclusion to be drawn from Table 1 is that it is not safe to store pears at a temperature below their juice freezing point. In no case was the true freezing point significantly higher than the juice freezing point, and in half the cases these two points were not significantly different. In a third of the cases, however, there is evidently no chance of freezing until the temperature falls about 1°F below the juice freezing point. The reason for this remains a matter for speculation. The validity of the assumption concerning the speed of the ice front which was postulated above in developing the theory has a bearing on this point and will be discussed in the next section.

TABLE 1
TEMPERATURE INTERVALS (°F) BY WHICH THE TRUE FREEZING POINT FELL BELOW THE JUICE FREEZING POINT

Time of Measurement	Variety of Pear Used				
	Beurre Bosc	Packham's Triumph	Peter Barry	Winter Cole	Winter Nelis
Within 1 month of picking	1.4	—	—	0.2	1.0,* 1.2
After at least 4 months in cool storage	0.3	0.1, 0	1.1, 0.4, 0.1	0.1	0.2,* 0.9

* See Figure 9.

VIII. THE SPEED OF THE ICE FRONT

The relation between the speed of the ice front and its temperature (eqn. (1)) is illustrated graphically in Figure 10, curve *W*. This particular expression was chosen partly in order to simplify the mathematics, but the broad agreement between the theory built on it and the experimental findings gives strong support to it. It is desirable, however, to examine the effect on the theoretical curves of adjusting the speed assumption in various ways. It appears likely that the general shape of the freezing and thawing curves would not be greatly altered by quite appreciable variations in the relation between the speed and the temperature. On the other hand the theoretical relation between initial freezing temperature and environment temperature (eqn. (7)) is quite sensitive to such variations. By reversing the procedure used above it is possible to deduce what the speed relation must be to give rise to any

particular curve relating initial freezing temperature and environment temperature. Various possibilities are shown in Figure 10. When the T_i versus T_0 line is 1°F lower (case X) the reason might be that some mechanism is lowering the freezing point of each cell by this amount. In this case the speed curve is also displaced 1°F downward, as at X_1 . If, however, the mechanism concerns the speed alone and not the latent heat released by each cell as it freezes, the speed relation X_2 must hold. If the T_i versus T_0 curve takes an upward turn at the end as at Y (the freezing point T being equal to the juice freezing point as in case W), the speed curve must also take an upward turn. Incidentally, if the speed curve W runs smoothly to zero as at Z this simply causes a local dip in the T_i versus T_0 curve as shown, without altering the extrapolated temperature T .

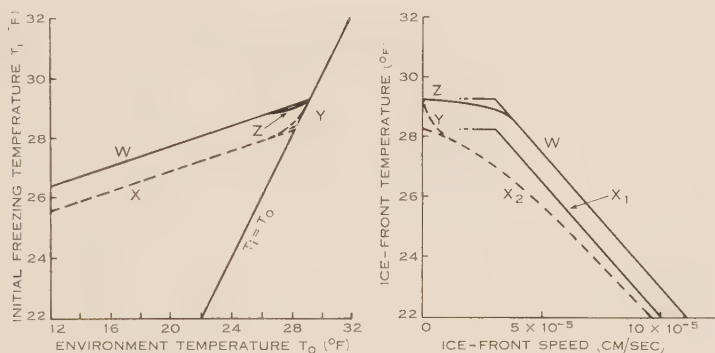


Fig. 10.—Various curves relating initial freezing temperature and environment temperature which would result from particular assumptions regarding the dependence of the speed of the ice front on its temperature. For explanation see text.

Theoretically one would expect any change in the speed curve to occur at the temperature where extracellular freezing gives way to intracellular freezing. In practice this transition occurred when the environment temperature T_0 was about 20 or 22°F , but the experiments carried out (Fig. 9) give no evidence of any behaviour like Figure 10, Y . Any conclusions, however, which are based on extrapolation as are those presented in Section VII can never be absolutely conclusive.

It may be possible to obtain direct experimental information about the temperature dependence of the ice-front speed. The ice front would be established by beginning the freezing process in the usual way, and then the temperature of the tissue held steady at the desired value by manipulating the environment temperature.

IX. DISCUSSION

It seems worth while to consider in more detail Jackson and Chalmers' (1958) treatment of the freezing of liquids in porous media, since this is not only the basis of the theory presented here but can also explain a number of points which are matters of dispute or mystery in the literature on the freezing of plant tissue.

They consider the various possible relative magnitudes of the interfacial free energies per unit area for the ice-substrate, water-substrate, and ice-water interfaces and conclude that when the substrate is soil the first of these free energies is not less than the sum of the latter two. In this case "the solid (ice) will tend to be separated from the substrate by a layer of liquid (water). In this latter case, the solid cannot nucleate at the substrate."

We may also conclude that the same situation holds when the substrate is plant tissue, since the high stability of cell sap in the supercooled state, which is emphasized by Asahina (1956) and is essential to our theory, indicates no tendency for ice to crystallize out on the surface of the tissue. Asahina's photomicrographs also show that when ice has formed, it does not adhere to the tissue but is surrounded by liquid in which it is frequently quite mobile.

Jackson and Chalmers then derive an expression for the depression of the freezing point of liquid in a capillary. This depression is inversely proportional to the radius of the capillary. For a radius of 0.1μ it is 0.2°C , for instance, and for a radius of 0.01μ , 2.0°C . They go on to point out that when a supercooled liquid is separated from its solid phase by a porous material with the described properties, the solid interface cannot propagate through the porous material, but the free energy of the system will be lowered if the liquid flows through the porous medium to the solid interface and freezes there. Now this is precisely the process which has been observed many times during the freezing of plant tissue. It can be seen that the smaller the size of the pores in the membrane or cell wall which hinders the advance of the ice front, the further must the liquid on the other side be supercooled before the liquid in the pores can freeze and allow the passage of the ice crystals into the supercooled liquid.

Asahina's attempt to explain the phenomenon in terms of the dendritic growth of the ice crystals as they "flash" into the supercooled liquid is inadequate. Dendritic growth is a dynamic effect, as Asahina himself indicates. The transmission of intracellular freezing in *Tradescantia*, however, is preceded by the slow growth of a "very clear ice mass"—the sharp crystals appear only on the far side of the barrier after penetration, and therefore can hardly be put forward as a cause of penetration. Chalmers (1959), incidentally, has written another more descriptive account of these various ways in which water freezes.

When, under constant-temperature conditions, an ice front encounters a porous membrane whose pores are too small to transmit ice crystals at that temperature, it may still be possible for the ice to penetrate the barrier if the membrane can be stretched under the pressure of the ice. Such a process has been observed by Modlibowska and Rogers (1955) and also by Asahina. An important point here is that additional energy is required to cause this stretching, and this is manifest as a depression of freezing point of the ice under the pressure of the stretched wall or membrane. This is the case discussed by Edlefsen and Anderson (1943, Section 30), where the pressure is exerted only on the ice and not on the water, resulting in a freezing point depression of 0.09°C per atmosphere of pressure. This depression is 12 times greater than is the case when the pressure acts equally on both ice and water.

If it happens that the freezing point is lowered in this way until it coincides with the prevailing temperature before the pores have become sufficiently enlarged to enable their contents to freeze, the process will come to a stop, unless the temperature is lowered further. As mentioned above, this explains why the temperature at which freezing can be propagated intracellularly is lower than that at which extracellular freezing may proceed.

If, on the other hand, the stretching proceeds till the second cell can be inoculated through the enlarged pores, the pressurized ice in the first cell will melt to the benefit of the unpressurized ice in the second cell, and the stretched cell wall will contract again—as has been observed experimentally.

In the case of “frost plasmolysis” described by Asahina the barrier membrane, although far more fragile than the cell wall, is protected from stretching by its flexibility, and provided the temperature does not fall below the critical value determined by the size of the pores, the state may continue indefinitely.

The theory is also capable of explaining the reduction in frost resistance caused by immersing cells in hypotonic solutions. Asahina found that there was a critical concentration of the solution below which the capacity of the cell to prevent the penetration of ice crystals into the cell interior was suddenly much reduced. He considers the possibility of “a swelling of the membrane in which the water space is so enlarged that the ice crystallisation in it is no longer disturbed”, but rejects this because the cell interior freezes by “flashing” which implies a considerable degree of supercooling. On the present theory, however, the swelling of the pores in the membrane is not regarded as removing all hindrance to ice penetration, but as steadily raising the temperature at which such penetration can occur. The weaker the hypotonic solution, the greater the quantity of water imbibed by the cell, and the greater the enlargement of the pores. For a particular temperature there will be a critical degree of stretching beyond which the water in the pores can freeze. But if extracellular freezing once begins at some part of the surface of such a cell, the resulting dehydration will immediately cause the pores to begin to shrink and remove any possibility of internal freezing. This is to be contrasted with the case of intracellular freezing considered above where the growth of ice causes further enlargement of the pores.

Conversely the action of hypertonic solutions in increasing frost resistance may be due to a reduction in pore size as well as a decrease in the amount of freezable water.

It may be remarked that in this theory the actual temperature is the important thing, not its rate of change. The preoccupation of much of the literature on the freezing of plant tissue with rates of cooling seems to be misleading. Due to the large size of the pieces of tissue used in the present experiments, the rates of cooling were never more than extremely slow according to the terminology suggested by Levitt (1958). In fact, after deep supercooling, intracellular freezing occurred even though the temperature was rising.

A further conclusion of the present theory is that the moisture in the surface of the cell walls will not freeze first as suggested by Levitt (1941, p. 182). Although

this water may be pure, it cannot have the same freezing point as free pure water, because its specific free energy would then be greater than the cell sap and it would immediately be imbibed into the cell by osmosis. Whereas the free energy of the cell sap is lowered by the solutes it contains, the free energy of the water in the cell wall is lowered by the surface energy effects which become dominant in very thin layers of liquid. Ice on the surface of the cell wall can come only from water extracted from the cell interior following some initial inoculation from elsewhere.

Partly for this reason, the objections which Levitt (1958, p. 21) raises against some of Aoki's interpretations (Aoki 1948, 1950) appear to be invalid. The second part of Levitt's objection is that he claims that rinsing pieces of tissue will effect the turgor of inner cells as well as those on the surface. Some of our own experiments, which admittedly used much larger pieces of tissue than Aoki's, tend to support Aoki's interpretation. These experiments were designed to investigate whether the apparent depression of freezing point during the course of freezing a whole pear might be due to a considerable migration of water from the unfrozen interior cells to the ice front. No evidence for such water movement was detected. A typical experiment was to cut one pear in pieces before freezing hard and thawing, while another similar pear was frozen whole and cut in pieces before thawing. The juice freezing points of tissue at various radii were then compared.

A complete answer to this point must involve a consideration of the rate of water diffusion in the particular tissue under a given free energy gradient in conjunction with the time between rinsing and measurement.

Concerning the differences between hardy and tender plants, one may make certain deductions about the structure of their cell walls or plasma membranes, in so far as the distinction between them lies in these members. It is generally agreed that hardy cells have a high permeability to water while at the same time being resistant to intracellular inoculation with ice. The latter implies pores of small radius, while the former would be favoured by a comparatively large number of pores of comparatively short length—i.e. a thin membrane. In tender cells, conversely, there must be a smaller number of longer and wider pores. Use of Jackson and Chalmers' formula in conjunction with careful observations like Asahina's, of the temperature at which a membrane allows transmission of ice crystals, should give useful information on the radius of the pores.

A puzzling feature of the present experiments has been the evidence that in some cases the true freezing point may be appreciably lower than the juice freezing point. The reason why such a difference should exist in the case of intracellular freezing has been presented, but this explanation can hardly apply to extracellular freezing since ice in the intercellular spaces, which are all interconnected, cannot be subject to much pressure. A possibility which might partially account for the effect is that the juice freezing point of the cells in the immediate vicinity of the initial freezing nucleus may be sufficiently lowered by the growth of ice at this nucleus to separate the ice from cells whose contents are supercooled. This, however, seems unlikely to account for more than a small fraction of a degree.

X. ACKNOWLEDGMENTS

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APPENDIX I

MATHEMATICAL DETAILS

(a) Derivation of Equation (2)

Disregarding the slight change in the absolute temperature, the depression of the freezing point of a solution is proportional to the mole fraction of dissolved material (e.g. Edlefsen and Anderson 1943, section 34). For a dilute solution the mole fraction is approximately proportional to $1/m$, where m is the quantity of water in which a given quantity of solute is dissolved. Thus

$$\text{Freezing point depression} = V_0 - v_F \propto 1/m.$$

Similarly $V_0 - V \propto 1/M$, where M is the original quantity of water before any has frozen. Therefore the fraction of water which freezes at temperature v_F is

$$\frac{M-m}{M} = 1 - \frac{m}{M} = 1 - \frac{V_0 - V}{V_0 - v_F} = \frac{V - v_F}{V_0 - v_F}.$$

(b) *The Freezing Equation*

We substitute in equation (5) the following expressions, which assume for simplicity that the density of the tissue both before and after freezing is 1.0 g/c.c.:

$$\text{Rate of heat loss at surface} = 4\pi a^2 H v_s \quad \text{cal/sec.}$$

$$\begin{aligned} \text{Rate of latent heat release at ice front} &= 4\pi \rho^2 S (V_0 - v_F) L \left(\frac{V - v_F}{V_0 - v_F} \right) \\ &= 4\pi \rho^2 L S (V - v_F) \quad \text{cal/sec.} \end{aligned}$$

$$\text{Right-hand side of equation (5)} = \frac{4}{3} \pi a^3 C \left(-\frac{dv_F}{dt} \right),$$

where C is the average specific heat of the pear. Equation (5) then becomes:

$$dv_F/dt = \{LS\rho^2(V - v_F) - Ha^2v_s\} / \frac{4}{3}a^3C, \quad (8)$$

and C is given by

$$C = 1 + \{(a^3 - \rho^3)/a^3\} L(V_0 - V)/(V_0 - v_{av})^2, \quad (9)$$

where v_{av} is an average temperature of the frozen shell. The first term represents the normal specific heat of the tissue, and the second term the effect of the marginal freezing and thawing in the frozen region.

It is necessary to express v_s and v_{av} in terms of the dependent variable v_F . To do this it was assumed for the moment that the net heat loss comes entirely from the frozen region, and uniformly from each point in it. This leads to the following expression for v_s :

$$v_s = \frac{K(a^2 + a\rho + \rho^2)v_F + LS\rho^2\{\frac{1}{2}(a + \rho) - a^2/\rho\}(V - v_F)}{K(a^2 + a\rho + \rho^2) + Ha^2\{\frac{1}{2}(a + \rho) - \rho^2/a\}}, \quad (10)$$

where K is the thermal conductivity of the frozen region. Although this is rather a cumbersome expression, the terms involving a and ρ can be tabulated once and for all at the beginning, and are not involved in the successive recalculations which are the most laborious aspect of a numerical solution.

Equation (10) agrees very closely with the exact solution which is available for the case $\rho = 0$ (Carslaw and Jaeger 1947, Section 92). The assumptions make it less accurate as ρ becomes larger, but at the same time $|v_F - v_s|$ becomes smaller so the absolute error should never be very large.

The expression for v_{av} is even more complicated than equation (10). However, the weighted mean

$$v_{av} = 0.4v_F + 0.6v_s, \quad (11)$$

is a good approximation to it at selected values of ρ , and has been used accordingly.

Equations (9), (10), and (11) enable the right-hand side of equation (8) to be expressed in terms of the two variables v_F and ρ . It is therefore desirable to use ρ rather than t as the independent variable. By putting

$$\frac{dv_F}{dt} = \frac{dv_F}{d\rho} \cdot \frac{d\rho}{dt} = \frac{dv_F}{d\rho} S(v_F - V_0),$$

equation (8) becomes

$$dv_F/d\rho = \{LS\rho^2(V-v_F) - Ha^2v_s\} / \frac{1}{3}a^3CS(v_F - V_0). \quad (12)$$

This equation can now be solved numerically to give v_F as a function of ρ . Equation (1) can then be integrated numerically to obtain v_F as a function of t as desired. Once the ice front reaches the centre, $\rho = 0$, and the equations are much simplified and can be solved analytically.

The four starting values for the numerical solution were obtained by using Picard's method of successive approximation (e.g. Kunz 1957, Section 8.13). The solution was then continued with an Adams predictor/corrector method (Kunz, Section 9.11). The interval h was taken as 0.05 cm initially and then increased to 0.10 cm at $\rho = 2.60$ cm, and to 0.20 cm at $\rho = 2.20$ cm. (The radius of the pear, a , was taken as 3.0 cm).

Since the main object of the calculation was to observe the general shape of the freezing curve, precise choosing of constants was considered unimportant. The latent heat L was arbitrarily taken as 70 cal/g, a value somewhat less than that for pure water. From a number of measurements of the rate of cooling of pears in the unfrozen state it was deduced that H was approximately 3×10^{-4} cal/cm² sec °C. The value 2.8×10^{-4} was actually used for arithmetical reasons. The speed factor S was chosen to make the time for the ice front to reach the centre about 8 hr as observed in practice when the environment temperature is low. Two values for S were tried, 2×10^{-5} and 4×10^{-5} cm/sec °C. The environment temperature was taken as 14°F, and the true freezing point as 29.3°F. In the units used this makes $V_0 = 10^\circ$ and $V = 8.5^\circ$. The thermal conductivity of the frozen region K was taken as 0.0050 c.g.s. units, slightly less than the value for pure ice, since even in the mildly frozen state the ice is assumed to form a connected network.

(c) *The Thawing Calculation*

In this case the method of finite differences was used. For radial heat flow in a sphere the differential equation is

$$\frac{\partial^2 v}{\partial r^2} + \frac{2}{r} \cdot \frac{\partial v}{\partial r} - \frac{1}{k} \cdot \frac{\partial v}{\partial t} = 0.$$

From this can be obtained the difference equation (cf. Kunz 1957, Section 14.1)

$$v_i^{j+1} = m(1+1/i)v_{i+1}^j + (1-2m)v_i^j + m(1-1/i)v_{i-1}^j,$$

where j is the number of time intervals of length p , and $r = ih$, and $m = kp/h^2$. In the frozen region the specific heat is

$$c = 1 + L(V_0 - V)/(V_0 - v)^2,$$

and since the diffusivity $k = K/\rho c$, where K and ρ are assumed to remain constant, it follows that as the temperature v rises the diffusivity decreases. To allow for this variation m was given the successive values 0.50, 0.48, 0.46, When the lowest temperature corresponded to $m = 0.40$ the time interval p was increased to raise this highest value of m to 0.50 again. The interval h was taken as 0.25 cm.

At the surface of the pear the boundary condition is now:

$$K(\partial v/\partial r)_{r=a} = H(V_0 - v_s),$$

since the environment is now at V_0 , the ice point. Using a differentiation formula involving three points this defines the surface temperature in terms of the two nearest interior points.

At the ice front it was assumed that heat flowed only into the frozen region. Since in the particular case calculated 70% of the pear was frozen when freezing began, and the frozen region had an equivalent specific heat of about 13 compared with unity in the unfrozen region, the error amounted to only a few per cent and decreased rapidly as the ice front advanced. The boundary condition is then:

$$K(\partial v/\partial r)_{r=\rho} = -LS(V - v_F).$$

The ice front was assumed to move in steps of $\frac{1}{4}h$, and four different formulas were used to define the temperature of the lattice point nearest to the ice front in terms of the temperatures of the two nearest points in the frozen region.

STUDIES IN TRANSLOCATION

I. THE RESPIRATION OF THE PHLOEM

By MARGARET D. DULOY* and F. V. MERCER*

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Summary

Comparative studies have been made of the respiratory metabolism of the vascular and ground tissues of the shoots of four species, *Apium graveolens*, *Cucurbita pepo*, *Vitis vinifera*, and *Verbascum virgatum*. As far as examined the respiration of the phloem tissue in each species was qualitatively identical with that of the surrounding ground tissues, and generally similar to that of other plant tissues. There was no evidence of a "peculiar metabolism" in the phloem; but the respiration rate of phloem tissue, per unit fresh weight, as measured by oxygen uptake, is calculated as being from 5 to 40 times higher than that of the ground parenchyma tissue of the same shoot. Since the differences in rates can be largely accounted for by differences in protein nitrogen, it is concluded that the higher respiration rates of the phloem are the result of its having more protoplasm per unit volume of tissue than the parenchyma.

I. INTRODUCTION

In recent years it has been suggested from studies with the light-microscope (Currier, Esau, and Cheadle 1955), and with the electron-microscope (Hepton, Preston, and Ripley 1955; Schumacher and Kollman 1959), that the sieve element on reaching anatomical, and it is assumed, physiological maturity, possesses a protoplast with possibly a peculiar metabolism which may be responsible for phloem transport. The high levels of enzymatic activity associated with the phloem tissue (Kuprevich 1949; Kendall 1955), and the very high respiration rates which have been calculated for the phloem (Kursanov and Turkina 1952; Kursanov, Turkina, and Dubenina 1953) have led the Russian workers to the conclusion that "sucrose or some substance to which it is readily converted in the conducting cells, is transported in the phloem by a process driven by the metabolic activity of the conducting cells" (Kursanov and Turkina 1952).

In view of the scarcity of information concerning phloem respiration, and since our own electron-microscope investigations (Duloy 1960; Duloy, Mercer, and Rathgeber 1961) do not support the conclusions drawn by other workers about the structure of the mature sieve-element protoplast, we have undertaken comparative studies of the respiration of phloem and ground tissues of four species. Most of the work was carried out using the stem of *Cucurbita pepo* (Duchesne), the mammoth cattle pumpkin. Some observations were made on tissues from the stem of *Vitis vinifera* (L.), the petiole of *Apium graveolens* (L.), and the midrib of the leaf of *Verbascum virgatum* (Stokes).

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II. MATERIALS AND METHODS

(a) *C. pepo*

Seeds were planted every 2 weeks during September–April in open plots, and stems were harvested 40–60 days from planting. The stem has a distinctive anatomy with two concentric cylinders, each of five bundles, the large outer bundles alternating with the small inner bundles. The pith is hollow. The bundles are bicollateral. A layer of cambiform tissue is present on either side of the xylem, but from studies with the light-microscope it was found that cambial activity is confined mainly to the outer side. In the sixth and older internodes a band of sclerenchyma is present in the pericycle. No further cambial activity takes place after the production of the sclerenchyma layer. Bundles in this condition are referred to as “mature”. Mature internodes are 15–20 cm in length, and 2–3 cm in diameter.

To harvest, a runner, including some 10 or 12 internodes, was cut from the vine, the cut end being placed in distilled water immediately. From this length of stem the first three mature internodes (sixth, seventh, and eighth) were taken separately and cut into lengths of 1 cm before being split vertically into two. The bundles of the inner ring were removed by two or three vertical cuts, and placed in a covered weighing bottle. The remaining stem tissue was trimmed so as to include only parenchyma, and placed in a second weighing bottle.

(b) *A. graveolens*

Material was obtained fresh from the market as required. This species was chosen because it is possible to isolate collenchyma from the petiole, as well as vascular tissue and parenchyma. The xylem consists mainly of vessels and xylem parenchyma. Abaxial to the phloem is a bundle cap composed of a type of collenchyma, less thickened than that in the cortex.

To prepare, the strips of cortical collenchyma and of vascular tissue were pulled separately away from the petiole and cut into 2-cm lengths. From the remainder of the petiole 2-cm segments of parenchyma were prepared.

(c) *V. vinifera*

Vines were grown in the open and were used for respiration studies between November and April. Canes some 50 cm in length were cut from the vine, the cut end being placed in distilled water. From this length internodes were cut one by one for treatment. The periderm was first peeled away exposing a layer of secondary phloem consisting of two comparatively thick crescents joined on either side by narrower strips. The crescents were cut into 1-cm lengths and slit into two.

(d) *V. virgatum*

The plants were growing in a loose rubble of ash and slag. When required, the plants were uprooted and the root systems were placed in distilled water. The leaves were removed one by one with a razor-blade. The vascular strand in the midrib was isolated by sharply bending the petiole and then pulling the petiole away from the

lamina. Both the xylem and phloem consists of radial rows of conducting cells* separated by bands of much-thickened collenchyma. Collenchyma also forms a bundle cap on either side of the bundle.

For all species, samples of 0.2–0.3 g fresh weight of each tissue were weighed from bulk lots in covered bottles, and either were transferred directly to Warburg vessels, or aerated overnight in water or unbuffered sucrose solutions. The composition of the vascular tissues as dissected are shown in Table 1. These values are used as the basis of calculations of the respiration rates of the phloem.

TABLE 1
PROPORTIONAL AREAS OF THE VARIOUS COMPONENTS OF THE VASCULAR TISSUES
OF FOUR SPECIES AS DISSECTED FOR RESPIRATION STUDIES

	Tissue	% of Total Tissue	% of Living Tissue
<i>C. pepo</i>	Lignified xylem*	22.2	0
	"Normal" parenchyma†	44.4	57
	Xylem parenchyma plus cambiform tissue	3.5	4.5
	Phloem	29.9	38.5
<i>A. graveolens</i>	Lignified xylem*	16.5	0
	Xylem parenchyma	16.8	20
	Cap collenchyma	44.3	53.2
	Phloem	22.4	26.8
<i>V. vinifera</i>	Phloem conducting cells‡	32.1	41
	Phloem fibres	21.4	0
	Rays	46.5	59
<i>V. virgatum</i>	Lignified xylem*	26	0
	Phloem conducting cells‡	22	30
	Phloem collenchyma	52	70

* i.e. includes vessels, tracheids, fibre-tracheids, and fibres.

† i.e. that which is identical and continuous with the surrounding ground parenchyma whose respiratory activity was measured.

‡ i.e. sieve tubes, companion cells, phloem parenchyma. Term used to distinguish these cells from phloem fibres or phloem collenchyma.

(e) Measurement of Respiration

Respiration rates were determined by measuring oxygen uptake at 25°C using the standard Warburg technique. The direct method of Warburg and Dixon was used to determine respiratory quotient (R.Q.) values. $\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$ buffers (M/30), with and without 0.03M sucrose, were used in all experiments unless otherwise noted. Organic acids were added as salts of potassium.

* i.e. vessels and tracheids in the xylem, sieve tubes, companion cells, and parenchyma in the phloem.

Except for the phloem of *V. vinifera* all the tissues, as dissected, contain some green cells but no effect of light on gas exchange was observed.

In all experiments the fresh weight of the tissues on cutting was used as the basis of calculation. The rates were expressed as $Q_{O_2}^F$, defined as oxygen uptake (in μ l) per gram fresh weight of tissue per hour.

Alcohol-insoluble nitrogen was estimated by the method of McKenzie and Wallace (1954). Total sugars were estimated by a modified Somogyi method.

Mitochondria were isolated from *C. pepo* in 0.4M sucrose in Tris-“Versene” buffer at pH 7.2, using standard centrifugation procedures at 0–2°C. The fraction isolated at 12,000 *g* for 30 min was used in these experiments.

Each of the experiments reported below was repeated at least three times.

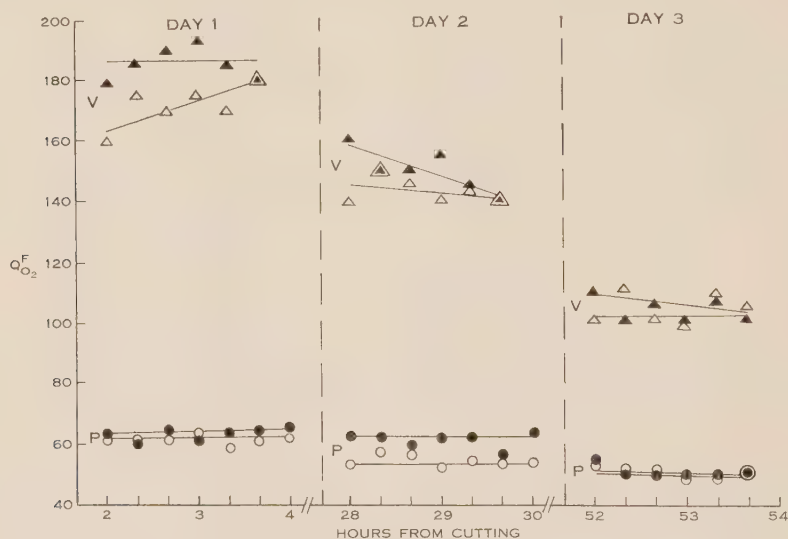


Fig. 1.—*C. pepo*: $Q_{O_2}^F$ values for vascular (V) and parenchyma (P) tissues stored in water at 2, 28, and 52 hr from cutting.

III. RESULTS

(a) *C. pepo*

(i) *Respiratory Levels*.— $Q_{O_2}^F$ values for vascular and parenchyma tissues over 2-hr periods at 3, 28, and 52 hr from cutting, are shown in Figure 1. The respiration rates fell with time, the decreases in the vascular tissue being greater than those of the parenchyma. The initial rate of the vascular tissue was two to three times as high as that of the parenchyma. Similar drifts were observed in ten experiments. Assuming (1) that lignified cells have no significant respiratory activity, (2) that the xylem parenchyma and cambiform tissue, which account for about 5% of the living tissue of the bundle, makes only a small contribution to the respiratory activity of the vascular tissue (the rate is taken to be equal to that of the “normal” parenchyma), and (3) that the normal parenchyma included with the vascular tissue is similar to

the ground parenchyma with which it is continuous, it follows that the rate of respiration of the phloem tissue must be considerably higher than that of the ground parenchyma. In the above experiment the $Q_{O_2}^F$ is calculated as being $540 \mu\text{l O}_2/\text{g}$ fresh weight/hr. i.e. nine times higher than that of the ground tissue. In 10 experiments differences of from approximately 5- to 15-fold were observed (Duloy 1960).

Two questions arise, (1) is the pathway of the high respiration of the phloem different from that of the low respiration of the ground tissue? and (2) does the presence of sucrose, which is thought to be the dominant mobile carbohydrate, affect the respiration of the phloem tissue?

(ii) *Response to Sucrose.*—The R.Q. values of both tissues stored in water ranged from 1.10 to 1.14 and remained unchanged over several days, and up to 90% of the loss of carbohydrate from the tissues could be accounted for by respiration.

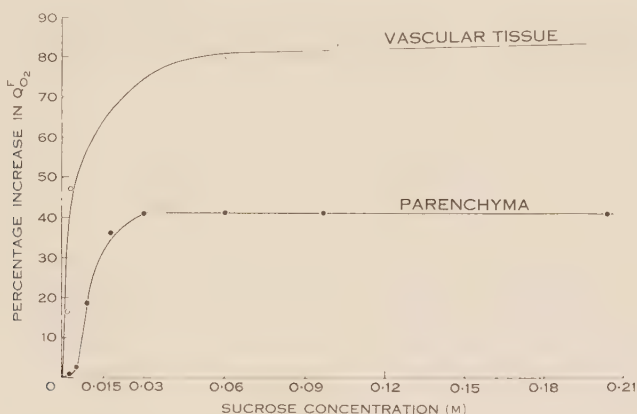


Fig. 2.—*C. pepo*: Percentage increases in $Q_{O_2}^F$ for vascular and parenchyma tissues with increasing concentrations of sucrose. Time from cutting 28 hr.

The respiration rates of both tissues increased with increasing concentrations of external sucrose up to 0.03M, no further increases being brought about by higher concentrations of sucrose (Fig. 2). The R.Q. values in the presence of 0.03M sucrose were almost identical with those in water, ranging from 1.10 to 1.22.

(iii) *Response to Intermediates of the Tricarboxylic Acid Cycle.*—Preliminary studies showed that stimulations in oxygen uptake of up to 25% were produced in both tissues by M/30 phosphate buffers at pH 5.0 and 6.5. Within this range, however, there appeared to be no effect of pH *per se* on the respiration of either tissue. From stimulations produced by the addition of M/30 KCl to tissue in unbuffered media, it was concluded that a salt effect due to potassium could account for about half of the stimulation produced by the buffer solutions. Since no further stimulations were produced by the addition of M/30 KCl to tissues in buffered solutions, it was concluded that a salt effect due to potassium did not make a significant contribution to the stimulations produced by the potassium salts of the intermediate acids.

As preliminary experiments showed that the respiration of both tissues attained steady rates and showed maximum response to treatment by day 2, experiments using intermediates and inhibitors were carried out with tissues 24–28 hr from cutting. Table 2 shows the response of the tissues to various intermediates of the tricarboxylic acid cycle. The addition of fumarate produced no response, but malate, succinate, and α -ketoglutarate increased the rate of oxygen uptake in both tissues, both in the presence and absence of sucrose, the effect being observed within 20 min of application. The R.Q. values rose only slightly, from 1.11–1.14 to 1.20–1.25, but an initial “gush” of CO_2 such as reported for other tissues (Turner and Hanly 1949) did not occur.

TABLE 2

C. PEPO: RESPIRATION OF VASCULAR AND PARENCHYMA TISSUES

Percentage increase in $Q_{\text{O}_2}^F$, over the rate in buffer or buffered sucrose, pH 5.0, produced by the addition of intermediates of the tricarboxylic acid cycle. Reaction mixtures contained: 0.25 g tissue, 4.0 ml M/30 phosphate buffer or buffered 0.03M sucrose, 0.45 ml intermediate, final concentration 0.05M. Time from cutting = 25 hr

	Malate	Succinate	α -Ketoglutarate
Vascular tissue in buffer	73	62	80
Vascular tissue in buffered sucrose	15	17	27
Parenchyma tissue in buffer	39	92	37
Parenchyma tissue in buffered sucrose	31	20	27

In all experiments with intermediates the respiration of the vascular tissue resembled that of the parenchyma, but had levels of activity two to three times greater. Values for the respiratory activity of the phloem calculated on the assumptions explained in the first section of the results, ranged from 550 to 1150 $\mu\text{l O}_2/\text{g}$ fresh weight/hr.

The addition of 0.02M malonate produced a 70–95% inhibition in the oxygen uptake of both tissues, in the presence and absence of 0.03M sucrose, within 1 hr of application. In the presence of 0.05M succinate, malate, or α -ketoglutarate, however, malonate caused an inhibition of only 10–15% in the oxygen uptake.

(iv) *Terminal Oxidase*.—The addition of 10^{-3}M KCN caused an 80–95% inhibition in the oxygen uptake of both tissues within 20 min of its application. The succinate-stimulated respiration was similarly inhibited. Finally the succinate-stimulated oxygen uptake of mitochondrial fractions from both tissues was further stimulated by the addition of cytochrome c (Fig. 3) indicating that the tissues possess an active cytochrome oxidase system.

(v) *Response to 2,4-Dinitrophenol*.—It was found that 10^{-5}M dinitrophenol produced a 65% stimulation in the oxygen uptake of the tissues in water, and a 25% stimulation in the tissues in 0.03M sucrose, within 20 min from the time of application.

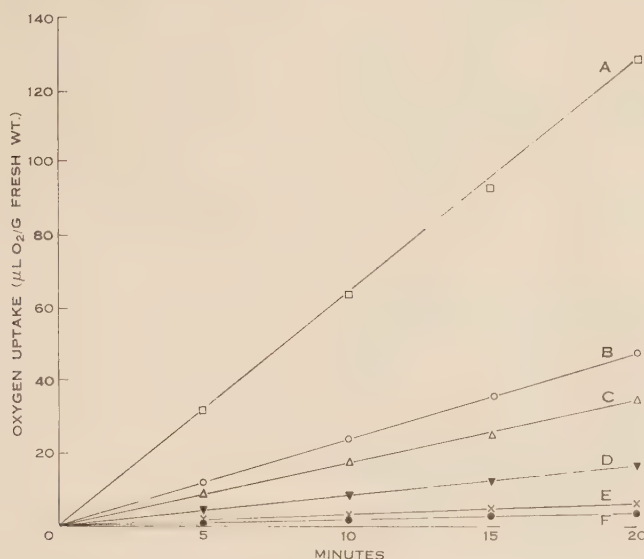


Fig. 3.—*C. pepo*: Oxidation of succinate and reduced cytochrome *c* by mitochondrial fractions from vascular and parenchyma tissues. The complete system contained: 1.5 ml mitochondrial suspension, 1.0 ml 1.6M sucrose, 0.4 ml 0.3M Tris- 0.28M acetate buffer, pH 7.2, 0.5 ml water, 0.4 ml cytochrome *c* ($60\text{ }\mu\text{M}$). A, vascular tissue plus cytochrome *c* plus succinate; B, vascular tissue plus succinate; C, parenchyma tissue plus cytochrome *c* plus succinate; D, parenchyma tissue plus succinate; E, parenchyma tissue plus cytochrome *c*; F, vascular tissue plus cytochrome *c*.

(b) *Tissues from other Species*

Rates of oxygen consumption for tissues from *V. vinifera*, *V. virgatum*, and *A. graveolens* under various conditions are shown in Table 3. In calculating the rates of oxygen consumption in the phloem, the following assumptions were made:

- (1) In *A. graveolens*, that the respiration rates of both the collenchymatous bundle cap of the vascular strand, and the xylem parenchyma, are equal to that of the cortical collenchyma.
- (2) In *V. virgatum*, that the respiration rate of the collenchyma is 1.5 times as high as that of the parenchyma, i.e. that the rates have the same ratio as is found for these tissues in *A. graveolens*.
- (3) In *V. vinifera*, that the fibres have no significant respiratory activity, and that the respiration rate of the ray parenchyma is equal to half that of the phloem conducting tissue.

So far as it was examined, the respiration of the phloem tissue of these species was qualitatively similar to that of the ground parenchyma tissues. In each species

TABLE 3
OXYGEN UPTAKE OF VASCULAR AND GROUND TISSUES OF THREE SPECIES

$Q_{O_2}^F$ values ($\mu l O_2/g$ fresh wt. tissue/hr) in presence and absence of 0.03M sucrose, under various conditions. Time from cutting 28 hr. Solutions used: M/30 phosphate buffer, pH 5.0; 0.05M succinate; $10^{-5}M$ 2, 4-dinitrophenol (DNP)

Tissue	Water	Sucrose	Water + DNP	Sucrose + DNP	Buffer	Buffer + Sucrose	Buffer + Succinate	Buffer + Sucrose + Succinate
<i>A. graveolens</i>								
Parenchyma	30	45	40	95	40	83	65	90
Collenchyma	70	90	100	130	55	108	170	230
Vascular	450	460	500	660	400	400	480	490
Phloem*	1820	1810	1960	2590	1690	1540	1730	1600
<i>V. virgatum</i>								
Parenchyma	70	130	100	190	65	130	115	180
Collenchyma*	140	260	200	380	130	260	230	360
Vascular	260	440	500	540	290	500	490	520
Phloem*	850	1380	1780	1550	870	1643	1670	1503
<i>V. vinifera</i>								
Phloem segment	230	360			340	450	450	600
Ray parenchyma*	210	256			310	410	410	545
Phloem conducting tissue*	420	512			620	820	820	1090

* Calculated values.

the respiratory activity of the phloem, per gram fresh weight, was calculated as being 10–50 times higher than that of ground parenchyma tissue.

There being no surrounding ground tissue in *V. vinifera*, measurements were carried out only on phloem segments. The rates recorded were higher than those of the ground tissues of the other species, and of storage organs which have been investigated (see Spector 1956). The R.Q. values for *V. vinifera* at pH 5.0 were found to be 0.8 in the absence of sucrose, 1.2 in its presence, and 2.5 in the presence of succinate. The last value suggests that in this tissue the oxidation of the added succinate did not proceed via the normal catalytic cycle. No R.Q. measurements were carried out for *A. graveolens* or *V. virgatum*.

TABLE 4
ALCOHOL-INSOLUBLE NITROGEN CONTENT FOR TISSUES OF
THREE OF THE SPECIES USED IN RESPIRATION STUDIES

Species	Nitrogen Content (mg/g fresh wt.)	
	Vascular Tissue	Parenchyma Tissue
<i>C. pepo</i>	1.07	0.35
<i>A. graveolens</i>	1.08	0.14
<i>V. virgatum</i>	1.96	0.50

(c) Protein Nitrogen

Protein (i.e. alcohol-insoluble) nitrogen were estimated for all species except *V. vinifera* (Table 4). The protein nitrogen content of the vascular tissue exceeded that of the parenchyma, so that in each species the respiration rate of the vascular tissue is found to be approximately equal to that of the parenchyma per unit weight of protein nitrogen.

IV. DISCUSSION

The results permit some general conclusions about the metabolism of the phloem tissue of *C. pepo*.

The observations (a) that the R.Q. values for both tissues in water are close to unity, and remain unchanged over long periods, (b) that approximately 90% of the loss of soluble carbohydrate from the tissues stored in water can be accounted for by respiration, (c) that the respiration rates are proportional to the external sucrose concentration up to 0.03M concentration, and (d) that the addition of sucrose produces little or no change in the R.Q. values, suggest that the primary substrate for respiration in both tissues is a soluble carbohydrate, and that this is in adequate supply, even in tissues without added sucrose, after several days.

The findings (i) that the oxygen uptake in both tissues is increased by the addition of α -ketoglutarate, succinate, and malate, (ii) that these increases are accom-

panied by only slight changes in R.Q., and (iii) that the malonate inhibition of oxygen uptake can be offset by succinate, α -ketoglutarate, and malate, together suggest that in both tissues respiration may proceed by a system generally similar to the tricarboxylic acid cycle.

Finally, from the findings (1) that the respiration of both tissues is extremely cyanide-sensitive, (2) that the succinate-stimulated respiration is similarly cyanide-sensitive, and (3) that the succinate-stimulated oxygen uptake of mitochondrial fractions from both tissues is further stimulated by the addition of cytochrome *c*, it seems likely that electron transfer in both tissues is mediated by the cytochrome oxidase system, to which is linked the succinic dehydrogenase system.

The stimulation of oxygen uptake by 2,4-dinitrophenol suggests that in both tissues some coupling exists between respiration and phosphorylation.

Collectively, the data indicate that the respiratory pathway in the phloem is identical with that in the parenchyma tissue, and generally similar to the pathways which have been found in other tissues, both plant and animal. That is, there is no evidence of a "unique" type of respiration either in the parenchyma tissue or in the phloem. Sucrose, which is regarded as the main mobile carbohydrate, is also respired by the phloem tissue, without inducing any alterations to the response of the tissue to respiratory intermediates and inhibitors, at least in isolated bundles. The results obtained for *V. vinifera*, *V. virgatum*, and *A. graveolens* support the conclusions reached for *C. pepo*.

Although the respiratory pathway seems to be identical in both vascular and parenchyma tissues in all four species, the respiratory activity of the phloem is apparently higher than that of the parenchyma, on a fresh weight basis, and may attain $Q_{O_2}^F$ values of almost 2000. These high values are consistent with the findings of Kursanov and Turkina (1952) and Kursanov, Turkina, and Dubenina (1953), who calculated $Q_{O_2}^F$ values of up to 5000 for the phloem of *Plantago*. Esau, Currier, and Cheadle (1957) have suggested that these high values may be overestimates because they were not corrected for the contribution of the xylem parenchyma and other living cells in the bundle. Even if such errors resulted in an overestimate of 100% the values would nevertheless remain very high as compared with storage tissue.

Recently Canny and Markus (1960) reported $Q_{CO_2}^F$ values of between 90 in water and 220 in sucrose for segments of phloem of *V. vinifera*, and concluded that phloem tissue does not have respiration rates significantly higher than those of ground or storage tissue. In contrast, in the present study $Q_{O_2}^F$ values of 230 in water to 450 in sucrose were recorded for phloem segments of *V. vinifera*, which after correcting for the possible contribution of the ray tissue gave calculated $Q_{O_2}^F$ values of the order of 1000 for the conducting tissue of the phloem. This estimate is dependent on the assumption that the respiration rate of the ray tissue is equal to half that of the phloem tissue.

There appear to be two groups of data concerning the respiration of the phloem, one indicating high rates, the other indicating low rates similar to those of parenchyma tissue. No explanation is offered of the differences between these groups of data. On the other hand an explanation of the high respiration rates calculated for

the phloem of three species described in this paper follows from a comparison of the protein nitrogen content of the tissues. Assuming protein nitrogen to be a measure of the protoplasmic content of a tissue, the higher activity of the phloem appears to be the result of its having more cytoplasm per unit volume of tissue than the parenchyma tissues.

The significance of the high respiratory activity of the phloem to the process of translocation, will be discussed in a later paper.

V. ACKNOWLEDGMENTS

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ACCOMMODATION OF GENE-CHROMOSOME CONFIGURATION EFFECTS IN QUANTITATIVE INHERITANCE AND SELECTION THEORY

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Summary

Gene-chromosome configuration effects may be generated in at least two different ways. The first results from the position-effect phenomenon, and the second, which is manifest if the individual is evaluated on the basis of its inbred progeny, is due to the restriction of independent segregation because of linkage. The present study is an attempt to generalize the gene model used in quantitative inheritance and selection theory so that it may accommodate these effects.

Configuration effects are defined and their relationships to the effects in the conventional model are examined for a random-mating population in equilibrium. Then, the expectations of various covariances among relatives are developed for the complete model which includes the configuration effects. Finally, the importance of this extension is discussed, primarily from the point of view of artificial selection.

I. INTRODUCTION

It is clear that more than one gene-chromosome arrangement is possible for diploid organisms heterozygous for the same set of genes at two or more linked loci. Thus, for two linked loci, the two possible genotypes are $(A_1^1 A_1^2) (A_2^1 A_2^2)$ and $(A_1^1 A_2^2) (A_2^1 A_1^2)$, where A_i^j is the i th allele at the j th locus, and the gene content within each set of parentheses indicates the association of genes within each homologous chromosome. More generally, if there are n such loci, the number of different genotypes for the given set of genes is 2^{n-1} .

From the standpoint of quantitative inheritance and selection theory, there are at least two ways in which the gene-chromosome arrangement may influence the evaluation of a genotype.

First, the physical configuration of the genes may induce position effects. The position-effect phenomenon was first discovered in *Drosophila melanogaster* by Sturtevant (1925), and more recently it has been found in a wide range of organisms.

Second, if the various gene arrangements are evaluated on the basis of their inbred progeny, it is obvious that different configurations may yield different evaluations for the same set of genes. For example, with the simplest situation involving only two loci, the expected selfed progeny arrays are different for the genotypes $(A_1^1 A_1^2) (A_2^1 A_2^2)$ and $(A_1^1 A_2^2) (A_2^1 A_1^2)$ if the recombination value is less than one-half. Hence, the expected means of these progeny arrays may be different if epistasis occurs.

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In quantitative inheritance theory no attempt has been made to accommodate effects due solely to the gene-chromosome arrangement. Hence, the objective of this study is to generalize the gene model used in quantitative inheritance so that it may do so. This requires both a genotypic representation which permits all possible gene-chromosome arrangements to be distinguishable, and a definition of configuration effects which may be incorporated into the gene model.

In the next section, an appropriate genotypic representation is developed; configuration effects are defined and their relationships to the effects in the conventional model are examined for a random-mating population in equilibrium. Then, the expectations of various covariances among relatives are developed for the complete model which includes the configuration effects. Finally, the importance of this extension is discussed, primarily from the point of view of artificial selection.

II. EXTENSION OF THE GENE MODEL

A random-mating population in equilibrium may be generated by multiplying the genotypic arrays for the various loci. Thus, for any number of alleles at each of two linked loci, the population may be represented as

$$\begin{aligned} \Pi &= (\sum_{ij} p_i^1 p_j^1 A_i^1 A_j^1) (\sum_{kl} p_k^2 p_l^2 A_k^2 A_l^2) \\ &= \sum_{ijkl} p_i^1 p_j^1 p_k^2 p_l^2 A_i^1 A_j^1 A_k^2 A_l^2, \end{aligned}$$

where

$$\sum_{ij} p_i^1 p_j^1 A_i^1 A_j^1 = \text{genotypic array at locus 1,}$$

and

$$\sum_{kl} p_k^2 p_l^2 A_k^2 A_l^2 = \text{genotypic array at locus 2.}$$

Kempthorne (1957 for general reference) has utilized this representation for the elaboration of his gene model. Thus, if d_{ijkl} = genotypic value for $A_i^1 A_j^1 A_k^2 A_l^2$, such that

$$\sum_{ijkl} p_i^1 p_j^1 p_k^2 p_l^2 d_{ijkl} = 0,$$

then the Kempthorne model may be set out as follows:

$$\begin{aligned} d_{ijkl} &= \alpha_i^1 + \alpha_j^1 + \alpha_k^2 + \alpha_l^2 + \delta_{ij}^1 + \delta_{kl}^2 + (\alpha\alpha)_{ik} + (\alpha\alpha)_{il} + (\alpha\alpha)_{jk} + (\alpha\alpha)_{jl} \\ &\quad + (\alpha\delta)_{ikl} + (\alpha\delta)_{jkl} + (\delta\alpha)_{ijk} + (\delta\alpha)_{ijl} + (\delta\delta)_{ijkl}, \end{aligned}$$

where

α_u^a = additive genetic effect of the A_u^a allele,

δ_{uv}^a = dominance effect for the $A_u^a A_v^a$ genotype,

$(\alpha\alpha)_{ik}$ = additive \times additive epistatic effect associated with genes A_i^1 and A_k^2 ,

$(\alpha\delta)_{ikl}$ = additive \times dominance epistatic effect associated with the gene A_i^1 and the genotype $A_k^2 A_l^2$, and

$(\delta\delta)_{ijkl}$ = dominance \times dominance epistatic effect associated with the genotypes $A_i^1 A_j^1$ and $A_k^2 A_l^2$.

This approach, however, does not permit the distinction between genotypic values for genotypes having the same set of genes but different chromosome arrangements. Hence, it is necessary to generate the population in such a way that the genotypes are represented by their chromosome constitution. This may be accomplished by simply squaring the chromosome array. For example, the population described above may be set out as follows:

$$\begin{aligned}\Pi &= [\sum_{ik} p_i^1 p_k^2 (A_i^1 A_k^2)]^2 \\ &= \sum_{ijkl} p_i^1 p_j^1 p_k^2 p_l^2 (A_i^1 A_k^2) (A_j^1 A_l^2),\end{aligned}$$

where

$$\sum_{ik} p_i^1 p_k^2 (A_i^1 A_k^2) = \text{chromosome array.}$$

The genotypic value of $(A_i^1 A_k^2) (A_j^1 A_l^2)$ may be designated as $d_{(ik)(jl)}$ such that

$$\sum_{ijkl} p_i^1 p_j^1 p_k^2 p_l^2 d_{(ik)(jl)} = 0.$$

In the remaining part of this section the argument will be concerned primarily with a random-mating population involving any number of alleles at each of two linked loci. Extensions to more than two loci will be briefly discussed at the end of the section.

(a) Development of Model for Two Linked Loci

The genotypic values in the two representations given above are related as follows:

$$d_{ijkl} = \frac{1}{2}(d_{(ik)(jl)} + d_{(il)(jk)}).$$

The inverse of this relationship leads to

$$\begin{aligned}d_{(ik)(jl)} &= d_{ijkl} + \frac{1}{2}(d_{(ik)(jl)} - d_{(il)(jk)}) \\ &= d_{ijkl} + c_{(ik)(jl)},\end{aligned}$$

where

$$\begin{aligned}c_{(ik)(jl)} &= \frac{1}{2}(d_{(ik)(jl)} - d_{(il)(jk)}) \\ &= \text{effect due to the difference generated by the different chromosome configurations.}\end{aligned}$$

It is clear that

$$\begin{aligned}c_{(il)(jk)} &= \frac{1}{2}(d_{(il)(jk)} - d_{(ik)(jl)}) \\ &= -c_{(ik)(jl)}.\end{aligned}$$

Various summations involving the c values are of interest in subsequent arguments. These are:

$$\begin{aligned}(1) \quad c_{(ik)(..)} &= \sum_j p_j^1 p_l^2 c_{(ik)(jl)} \\ &= -\sum_k p_k^1 p_l^2 c_{(il)(jk)} \\ &= -c_{(i..)(..k)}. \\ (2) \quad c_{(i..)(..)} &= \sum_j p_j^1 p_k^2 p_l^2 c_{(ik)(jl)} \\ &= -\sum_j p_j^1 p_k^2 p_l^2 c_{(il)(jk)} \\ &= -c_{(i..)(..)}.\end{aligned}$$

Hence

$$\begin{aligned}
 c_{(i.)(.)} &= 0. \\
 (3) \quad c_{(i.)(.)} &= \sum p_k^2 p_l^2 c_{(ik)(jl)} \\
 &= -\sum p_k^2 p_l^2 c_{(il)(jk)} \\
 &= -c_{(i.)(.)} .
 \end{aligned}$$

Hence

$$c_{(i.)(.)} = 0.$$

The properties of $c_{(ik)(jl)}$, for the random-mating population, are as follows (where E denotes the expectation over i, j, k , and l):

$$\begin{aligned}
 E(c_{(ik)(jl)}) &= \sum_{ijkl} p_i^1 p_j^1 p_k^2 p_l^2 c_{(ik)(jl)} \\
 &= \frac{1}{2} [\sum_{ijkl} p_i^1 p_j^1 p_k^2 p_l^2 (d_{(ik)(jl)} - d_{(il)(jk)})] \\
 &= 0.
 \end{aligned}$$

Also

$$\begin{aligned}
 E(c_{(ik)(jl)})^2 &= \sum_{ijkl} p_i^1 p_j^1 p_k^2 p_l^2 (c_{(ik)(jl)})^2 \\
 &= \sigma_C^2.
 \end{aligned}$$

Since $d_{(ik)(jl)} = d_{ijkl} + c_{(ik)(jl)}$, it is desirable to show that $c_{(ik)(jl)}$ is independent of d_{ijkl} . This may be accomplished in the following manner:

$$\begin{aligned}
 E(d_{ijkl} \cdot c_{(ik)(jl)}) &= \sum_{ijkl} p_i^1 p_j^1 p_k^2 p_l^2 [d_{ijkl} \cdot c_{(ik)(jl)}] \\
 &= \frac{1}{4} [\sum_{ijkl} p_i^1 p_j^1 p_k^2 p_l^2 (d_{(ik)(jl)} + d_{(il)(jk)}) (d_{(ik)(jl)} - d_{(il)(jk)})] \\
 &= \frac{1}{4} [\sum_{ijkl} p_i^1 p_j^1 p_k^2 p_l^2 (d_{(ik)(jl)}^2 - d_{(il)(jk)}^2)] \\
 &= 0.
 \end{aligned}$$

Although $c_{(ik)(jl)}$ is independent of d_{ijkl} , and therefore independent of entire classes of elements in the gene model, it need not be independent of some of the individual elements. The following considers the expectations of cross-products of $c_{(ik)(jl)}$ with entire classes of effects as well as with individual component elements of the gene model.

(1) Additive Effects

$$\begin{aligned}
 E[c_{(ik)(jl)} \cdot (\alpha_i^1 + \alpha_j^1 + \alpha_k^2 + \alpha_l^2)] &= \sum_i p_i^1 c_{(i.)(.)} \alpha_i^1 + \sum_j p_j^1 c_{(.)(j.)} \alpha_j^1 \\
 &\quad + \sum_k p_k^2 c_{(.)(k.)} \alpha_k^2 + \sum_l p_l^2 c_{(.)(l.)} \alpha_l^2 \\
 &= 0,
 \end{aligned}$$

since

$$c_{(i.)(.)} = c_{(.)(j.)} = c_{(.)(k.)} = c_{(.)(l.)} = 0.$$

(2) Dominance Effects

$$\begin{aligned}
 E[c_{(ik)(jl)} \cdot (\delta_{ij}^1 + \delta_{kl}^2)] &= \sum_{ij} p_i^1 p_j^1 c_{(i.)(.)} \delta_{ij}^1 + \sum_{kl} p_k^2 p_l^2 c_{(.)(k.)} \delta_{kl}^2 \\
 &= 0,
 \end{aligned}$$

since

$$c_{(i.)(.)} = c_{(.)(k.)} = 0.$$

(3) *Additive \times Additive Effects*

$$\begin{aligned}
E\{c_{(ik)(jl)} \cdot [(aa)_{ik} + (aa)_{il} + (aa)_{jk} + (aa)_{jl}]\} \\
= \sum_{ik} p_i^1 p_k^2 c_{(ik)(..)} (aa)_{ik} + \sum_{il} p_i^1 p_l^2 c_{(i..)(.l)} (aa)_{il} \\
+ \sum_{jk} p_j^1 p_k^2 c_{(.k)(j..)} (aa)_{jk} + \sum_{jl} p_j^1 p_l^2 c_{(..)(jl)} (aa)_{jl} \\
= [\sum_{ik} p_i^1 p_k^2 c_{(ik)(..)} (aa)_{ik} - \sum_{il} p_i^1 p_l^2 c_{(il)(..)} (aa)_{il}] \\
+ [\sum_{jl} p_j^1 p_l^2 c_{(..)(jl)} (aa)_{jl} - \sum_{jk} p_j^1 p_k^2 c_{(..)(jk)} (aa)_{jk}] \\
= 0.
\end{aligned}$$

The fact that the difference within each bracket obviously equals zero does not imply that the individual terms within the brackets equal zero. Hence quantities of the type

$$\sum_{ik} p_i^1 p_k^2 c_{(ik)(..)} (aa)_{ik},$$

need not equal zero.

(4) *Additive \times Dominance Effects*

$$\begin{aligned}
E\{c_{(ik)(jl)} \cdot [(a\delta)_{ikl} + (a\delta)_{jkl}]\} &= \sum_{ikl} p_i^1 p_k^2 p_l^2 c_{(ik)(.l)} (a\delta)_{ikl} + \sum_{jkl} p_j^1 p_k^2 p_l^2 c_{(.k)(jl)} (a\delta)_{jkl} \\
&= A + B \\
&= 0.
\end{aligned}$$

The term A is equal to zero, since for each combination of alleles A_i^1 , A_k^2 , and A_l^2 there are two c configurations (due to the interchange of alleles at the A^2 locus) with the same frequency. These configurations are equal in magnitude but differ in sign, i.e.

$$c_{(ik)(.l)} = -c_{(il)(.k)}.$$

However, the interchange of A_k^2 and A_l^2 does not alter the value of $(a\delta)_{ikl}$. Hence, the cross-product contributions involving A_i^1 , A_k^2 , and A_l^2 are

$$\begin{aligned}
p_i^1 p_k^2 p_l^2 [c_{(ik)(.l)} (a\delta)_{ikl} + c_{(il)(.k)} (a\delta)_{ilk}] &= p_i^1 p_k^2 p_l^2 [c_{(ik)(.l)} (a\delta)_{ikl} - c_{(ik)(.l)} (a\delta)_{ikl}] \\
&= 0.
\end{aligned}$$

Similarly, the term B is equal to zero.

(5) *Dominance \times Additive Effects*

An argument similar to that given in (4) is applicable.

(6) *Dominance \times Dominance Effects*

$$\begin{aligned}
E[c_{(ik)(jl)} \cdot (\delta\delta)_{ijkl}] &= \sum_{ijkl} p_i^1 p_j^1 p_k^2 p_l^2 c_{(ik)(jl)} \cdot (\delta\delta)_{ijkl} \\
&= 0.
\end{aligned}$$

That this summation equals zero can be seen by considering all possible permutations of each combination of A_i^1 , A_j^1 , A_k^2 , and A_l^2 , which are generated by inter-

changing A_i^1 and A_j^1 as well as A_k^2 and A_l^2 . Such operations may change the value of the c values but do not alter the values of the $(\delta\delta)$ terms. Hence, the cross-product contribution of the combination $A_i^1 A_j^1 A_k^2 A_l^2$ is

$$\begin{aligned} p_i^1 p_j^1 p_k^2 p_l^2 [c_{(ik)(jl)} (\delta\delta)_{ijkl} + c_{(jk)(il)} (\delta\delta)_{jikl} + c_{(il)(jk)} (\delta\delta)_{ijlk} + c_{(jl)(ik)} (\delta\delta)_{jilk}] \\ = p_i^1 p_j^1 p_k^2 p_l^2 [2c_{(ik)(jl)} - 2c_{(ik)(jl)}] (\delta\delta)_{ijkl} \\ = 0. \end{aligned}$$

In summary then, the gene model for the genotype $(A_i^1 A_k^2) (A_j^1 A_l^2)$ is

$$\begin{aligned} d_{(ik)(jl)} = & \alpha_i^1 + \alpha_j^1 + \alpha_k^2 + \alpha_l^2 + \delta_{ij}^1 + \delta_{kl}^2 + (\alpha\alpha)_{ik} + (\alpha\alpha)_{il} + (\alpha\alpha)_{jk} + (\alpha\alpha)_{jl} \\ & + (\alpha\delta)_{ikl} + (\alpha\delta)_{jkl} + (\delta\alpha)_{ijk} + (\delta\alpha)_{ijl} + (\delta\delta)_{ijkl} + c_{(ik)(jl)}, \end{aligned}$$

where all effects are independent of each other except that the individual $(\alpha\alpha)$'s are not independent of $c_{(ik)(jl)}$. The total genotypic variance may be partitioned as:

$$\sigma^2 = \sigma_A^2 + \sigma_D^2 + \sigma_{AA}^2 + \sigma_{AD}^2 + \sigma_{DD}^2 + \sigma_C^2.$$

(b) *Extension of Model to more than Two Linked Loci*

In extending the theory to more than two linked loci, the first problem is to determine the number of different genotypes which are possible by permuting the two alleles at each of an arbitrary number of loci.

For the a th locus with alleles A_i^a and A_j^a the arrangements may be generated by a permutation group of order two, i.e. $[G = I, (ij)]$. Hence, for n loci, all possible arrangements may be obtained by application of an Abelian permutation group of order 2^n which results as a direct product of the n permutation groups of order two.

However, not all gene-chromosome arrangements give rise to different genotypes, since genotypes are invariant to permutation of chromosomes. In fact, the 2^n permutations may be paired such that in one member of a pair, alleles at all loci are interchanged which are not permuted in the other. Such pairs of permutations generate equivalent arrangements, one of which may be derived from the other by chromosome interchange. Hence, the number of different genotypes is 2^{n-1} .

The following tabulation presents the simplest illustration of the above argument. It gives the permutation group, gene-chromosome arrangements, and genotypes which are possible for two linked heterozygous loci.

Permutation Group	Gene-Chromosome Arrangements	Genotypes
I	$(A_i^1 A_k^2) (A_j^1 A_l^2)$	$(A_i^1 A_k^2) (A_j^1 A_l^2) = (A_j^1 A_l^2) (A_i^1 A_k^2)$
(ij)	$(A_j^1 A_k^2) (A_i^1 A_l^2)$	$(A_j^1 A_k^2) (A_i^1 A_l^2) = (A_i^1 A_l^2) (A_j^1 A_k^2)$
(kl)	$(A_i^1 A_l^2) (A_j^1 A_k^2)$	
$(ij) (kl)$	$(A_j^1 A_l^2) (A_i^1 A_k^2)$	

Finally, there are a total of 2^{n-1} configuration constants for genotypes heterozygous for the same set of n linked loci, since a constant is associated with each

genotype. However, since these constants must sum to zero, the number of independent effects is $2^{n-1}-1$.

As an illustrative example, consider the situation which arises for three linked loci. The four possible genotypes are: $(A_i^1 A_k^2 A_m^3)$ $(A_j^1 A_l^2 A_n^3)$, $(A_j^1 A_k^2 A_m^3)$ $(A_i^1 A_l^2 A_n^3)$, $(A_i^1 A_l^2 A_m^3)$ $(A_j^1 A_k^2 A_n^3)$, and $(A_i^1 A_k^2 A_n^3)$ $(A_j^1 A_l^2 A_m^3)$.

Since

$$d_{ijkilmn} = \frac{1}{4}[d_{(ikm)(jln)} + d_{(jkm)(iln)} + d_{(ilm)(jkn)} + d_{(ikn)(jlm)}],$$

then

$$\begin{aligned} d_{(ikm)(jln)} &= d_{ijkilmn} + c_{(ikm)(jln)}, \\ d_{(jkm)(iln)} &= d_{ijkilmn} + c_{(jkm)(iln)}, \text{ etc.}, \end{aligned}$$

where

$$\begin{aligned} c_{(ikm)(jln)} &= \frac{1}{4}[(d_{(ikm)(jln)} - d_{(jkm)(iln)}) + (d_{(ikm)(jln)} - d_{(ilm)(jkn)}) \\ &\quad + (d_{(ikm)(jln)} - d_{(ikn)(jlm)})], \\ c_{(jkm)(iln)} &= \frac{1}{4}[(d_{(jkm)(iln)} - d_{(ikm)(jln)}) + (d_{(jkm)(iln)} - d_{(ilm)(jkn)}) \\ &\quad + (d_{(jkm)(iln)} - d_{(ikn)(jlm)})], \\ c_{(ilm)(jkn)} &= \frac{1}{4}[(d_{(ilm)(jkn)} - d_{(ikm)(jln)}) + (d_{(ilm)(jkn)} - d_{(jkm)(iln)}) \\ &\quad + (d_{(ilm)(jkn)} - d_{(ikn)(jlm)})], \end{aligned}$$

and

$$\begin{aligned} c_{(ikn)(jlm)} &= \frac{1}{4}[(d_{(ikn)(jlm)} - d_{(ikm)(jln)}) + (d_{(ikn)(jlm)} - d_{(jkm)(iln)}) \\ &\quad + (d_{(ikn)(jlm)} - d_{(ilm)(jkn)})]. \end{aligned}$$

However, since the following linear restriction holds, there are only three independent constants:

$$c_{(ikm)(jln)} + c_{(jkm)(iln)} + c_{(ilm)(jkn)} + c_{(ikn)(jlm)} = 0.$$

From this brief discussion, it is clear that it is conceptually possible to define and enumerate configuration effects for any number of linked loci.

III. EXPECTATIONS OF COVARIANCES AMONG RELATIVES

Estimation of the additive variance component is essential if the permanent gains from selection are to be predicted. In the past, various covariances among relatives have been used to make this estimation. The covariances of interest are: parent-offspring covariance, designated as Cov(PO); half-sib covariance, designated as Cov(HS); and full-sib covariance, designated as Cov(FS).

The objective of this section is to develop the expectations of these covariances for a two-locus gene model which is generalized to include the following details:

- (i) any number of alleles at each locus;
- (ii) any system of dominance and epistatic parameters;
- (iii) recombination values which may be different for the two sexes, i.e.

y_m = recombination value for males, and

y_f = recombination value for females; and

- (iv) gene-chromosome configuration effects.

Since the expectations of the covariances have been derived for a gene model which is generalized for all but the inclusion of configuration effects (Griffing 1960*b*), the purpose, here, is to see how these configuration effects disturb the covariances, and hence the estimation of variance components from these covariances.

The parent-offspring covariance may be defined as the expected cross-product of the genotypic value of an arbitrary parent individual and the genotypic mean of the half-sib array associated with the parent individual. If configuration constants are not considered, it can be shown that linkage does not disturb $\text{Cov}(\text{PO})$. However, when these effects are included, not only does linkage disturb this covariance but the covariance for males may be different from that for females. Therefore, they must be treated separately. Consider first the male covariance which may be designated as $\text{Cov}_{(m)}(\text{PO})$.

An arbitrary male ($A_i^1 A_k^2$) ($A_j^1 A_l^2$) produces the following gametic array:

$$\{[(1-y_m)/2] (A_i^1 A_k^2 + A_j^1 A_l^2) + (y_m/2) (A_i^1 A_l^2 + A_j^1 A_k^2)\}.$$

The total female gametic array for the random-mating population is

$$\sum_{rt} p_r^1 p_t^2 (A_r^1 A_t^2).$$

Therefore, the male half-sib family mean is

$$\begin{aligned} h_{(ik, jl) (\dots)} &= [(1-y_m)/2] \sum_{rt} p_r^1 p_t^2 [d_{(ik)(rt)} + d_{(jl)(rt)}] \\ &\quad + (y_m/2) \sum_{rt} p_r^1 p_t^2 [d_{(il)(rt)} + d_{(jk)(rt)}] \\ &= [(1-y_m)/2] (d_{(ik)(..)} + d_{(jl)(..)}) + (y_m/2) (d_{(il)(..)} + d_{(jk)(..)}). \end{aligned}$$

The male parent-offspring covariance is then

$$\begin{aligned} \text{Cov}_{(m)}(\text{PO}) &= \sum_{ijkl} p_i^1 p_j^1 p_k^2 p_l^2 (h_{(ik, jl) (\dots)}) (d_{(ik)(jl)}) \\ &= \frac{1}{2} \sigma_A^2 + \frac{1}{4} \sigma_{AA}^2 + (1-2y_m) \sum_{ik} p_i^1 p_k^2 (c_{(ik)(..)})^2 \\ &\quad + 2(1-y_m) \sum_{ik} p_i^1 p_k^2 (c_{(ik)(..)}) (aa)_{ik}. \end{aligned}$$

Similarly, the female parent-offspring covariance is

$$\begin{aligned} \text{Cov}_{(f)}(\text{PO}) &= \frac{1}{2} \sigma_A^2 + \frac{1}{4} \sigma_{AA}^2 + (1-2y_f) \sum_{ik} p_i^1 p_k^2 [c_{(ik)(..)}]^2 \\ &\quad + 2(1-y_f) \sum_{ik} p_i^1 p_k^2 [c_{(ik)(..)}] (aa)_{ik}. \end{aligned}$$

The half-sib covariance may be defined as the expectation of the squares of the half-sib family means. Again, male and female half-sib covariances may be different. Consider, first, the derivation of the male half-sib covariance.

Since the half-sib family mean for an arbitrary male ($A_i^1 A_k^2$) ($A_j^1 A_l^2$) has been given, it is obvious that the male covariance of half-sibs is

$$\begin{aligned} \text{Cov}_{(m)}(\text{HS}) &= \sum_{ijkl} p_i^1 p_j^1 p_k^2 p_l^2 (h_{(ik, jl)(\dots)})^2 \\ &= \frac{1}{4} \sigma_A^2 + [(1 + \delta_m)/16] \sigma_{AA}^2 + [(1 + \delta_m)/4] \sum_{ik} p_i^1 p_k^2 (c_{(ik)(\dots)})^2 \\ &\quad + [(1 + \delta_m)/2] \sum_{ik} p_i^1 p_k^2 (c_{(ik)(\dots)}) (\alpha\alpha)_{ik}, \end{aligned}$$

where

$$\delta_m = (1 - 2y_m)^2.$$

Likewise, the female half-sib covariance is

$$\begin{aligned} \text{Cov}_{(f)}(\text{HS}) &= \sum_{rstu} p_r^1 p_s^1 p_t^2 p_u^2 (h_{(\dots)(rt, su)})^2 \\ &= \frac{1}{4} \sigma_A^2 + [(1 + \delta_f)/16] \sigma_{AA}^2 + [(1 + \delta_f)/4] \sum_{rt} p_r^1 p_t^2 (c_{(\dots)(rt)})^2 \\ &\quad + [(1 + \delta_f)/2] \sum_{rt} p_r^1 p_t^2 (c_{(\dots)(rt)}) (\alpha\alpha)_{rt}, \end{aligned}$$

where

$$\delta_f = (1 - 2y_f)^2.$$

Finally, the full-sib covariance may be defined as the expected value of the squares of the full-sib means. Consider, now, the evaluation of $\text{Cov}(\text{FS})$.

The mean of the full-sib array which results from the cross between an arbitrary male ($A_i^1 A_k^2$) ($A_j^1 A_l^2$) and an arbitrary female ($A_r^1 A_t^2$) ($A_s^1 A_u^2$) is

$$\begin{aligned} h_{(ik, jl)(rt, su)} &= \{[(1 - y_m)/2][(1 - y_f)/2](d_{(ik)(rt)} + d_{(ik)(su)} + d_{(jl)(rt)} + d_{(jl)(su)}) \\ &\quad + [(1 - y_m)/2](y_f/2)(d_{(ik)(ru)} + d_{(ik)(st)} + d_{(jl)(ru)} + d_{(jl)(st)}) \\ &\quad + (y_m/2)[(1 - y_f)/2](d_{(il)(rt)} + d_{(il)(su)} + d_{(jk)(rt)} + d_{(jk)(su)}) \\ &\quad + (y_m/2)(y_f/2)(d_{(il)(ru)} + d_{(il)(st)} + d_{(jk)(ru)} + d_{(jk)(st)})\}. \end{aligned}$$

The covariance of full-sibs is then

$$\begin{aligned} \text{Cov}(\text{FS}) &= \sum_{ijklrstu} p_i^1 p_j^1 p_r^1 p_s^1 p_k^2 p_l^2 p_t^2 p_u^2 (h_{(ik, jl)(rt, su)})^2 \\ &= \frac{1}{2} \sigma_A^2 + \frac{1}{4} \sigma_D^2 + \left\{ \frac{1}{4} + [(\delta_f + \delta_m)/16] \right\} \sigma_{AA}^2 + \left\{ \frac{1}{8} + [(\delta_f + \delta_m)/16] \right\} \sigma_{AD}^2 \\ &\quad + \left[\frac{1}{16} (1 + \delta_f)(1 + \delta_m) \right] \sigma_{DD}^2 + \left[\frac{1}{16} (1 + \delta_f)(1 + \delta_m) \right] \sigma_C^2 \\ &\quad + \left[\frac{1}{8} (1 + \delta_f)(1 + \delta_m) + 2(y_m)(y_f)(1 - y_m)(1 - y_f) \right] \sum_{ik} p_i^1 p_k^2 (c_{(ik)(\dots)})^2 \\ &\quad + \{[y_f(1 - y_f)/4](1 + \delta_m) + [y_m(1 - y_m)/4](1 + \delta_f)\} \\ &\quad \times \left\{ \sum_{irk} p_i^1 p_r^1 p_k^2 (c_{(ik)(r, \dots)})^2 + \sum_{ikl} p_i^1 p_k^2 p_l^2 (c_{(ik)(\dots, l)})^2 \right\} \\ &\quad + \left[\frac{1}{4} (1 + \delta_f)(1 + \delta_m) - 4(y_m)(y_f)(1 - y_m)(1 - y_f) \right] \sum_{ik} p_i^1 p_k^2 (c_{(ik)(\dots)}) (\alpha\alpha)_{ik}. \end{aligned}$$

Assuming (i) epistatic interactions involving three or more loci are negligible, and (ii) configuration effects are absent, it has been shown (Griffing 1960b) that σ_A^2 and σ_{AA}^2 can be estimated as follows:

$$\sigma_A^2 = \frac{\text{Cov}(\text{PO})[2\bar{y}_m(1 - \bar{y}_m) - 1] + 2[\text{Cov}_{(m)}(\text{HS})]}{\bar{y}_m(1 - \bar{y}_m)},$$

and

$$\hat{\sigma}_{AA}^2 = \frac{2\{\text{Cov}(\text{PO}) - 2[\text{Cov}_{(m)}(\text{HS})]\}}{\bar{y}_m(1 - \bar{y}_m)},$$

where \bar{y}_m represents the recombination value averaged over all possible pairs of active loci as measured in the male sex.

However, it is now clear that if the configuration effects are taken into consideration, these variance component estimates are no longer unbiased.

IV. DISCUSSION

The question of how far and in what way the inclusion of configuration effects may disturb prediction theory is discussed below.

Earlier it was pointed out that there are at least two ways in which configuration effects may be generated. First, the physical configuration of the genes may induce the position-effect phenomenon. Second, linkage may give rise to configuration effects if the various gene-chromosome arrangements are evaluated on the basis of their inbred progeny. These different sources of disturbance are discussed separately.

A discussion of the position-effect phenomenon necessitates a brief consideration of the modern concept of the "gene". This concept postulates that the chromosome may be divided into functional regions each of which controls a specific biological activity. These functional regions may each contain numerous mutational and recombinational sites. In some cases it has been shown that linear linkage maps may be obtained from intralocus recombination data. Mutations are assigned to functional regions on the basis of the position-effect criterion, the site of the mutation being the muton (see Benzer 1957). Thus, mutations exhibiting position effect are assigned to the same functional region and those that do not exhibit this effect are assigned to different regions.

From this concept of the gene two points need to be considered with regard to the importance of the position-effect phenomenon in prediction theory. First, there are at least two possibilities in the choice of a basic hereditary unit on which the selection theory rests. Second, position effects are usually generated by mutations which are very close together, i.e. mutations in the same functional region.

The choice of a basic hereditary unit has been discussed previously (Griffing 1960a):

"There are at least two methods of representing the genetic situation at a complex locus. To illustrate, consider a locus which has a simplified structure consisting of only two genetic conditions (mutant and normal) at each of two mutational sites. In the first method, the locus can be subdivided into two subloci, one for each of the mutational sites. This approach yields two sets of alleles, each set being the genetic alternatives at each sublocus. In this case, the gene model for quantitative inheritance must be extended to accommodate position effect which may occur between alleles at different subloci. This, so far, has never been done.

The alternative method is to consider the overall locus as the basic entity, and to regard all possible genetic structures at this locus as the set of multiple alleles. Thus, in the simplified example, the four possible gene states are $(+ +)$, $(m_1 +)$, $(+ m_2)$, and $(m_1 m_2)$. These, then, would be regarded as the alleles of the locus. Such a representation avoids the introduction of intralocus position effect because complexities such as the *cis-trans* relations would be absorbed

in the dominance parameters. However, a resultant complication of this approach is that mutation of alleles as defined above includes both point mutation in its conventional sense and intralocus recombination. For example, recombination between mutational sites arranged in a *trans* configuration, $m_1 +/+ m_2$, results in non-parental locus types, $(+ +)$ and $(m_1 m_2)$. It is of course clear that the frequency of such intralocus recombination is low compared with the frequency of recombination between genes at different loci. Hence, it would appear that with the alleles defined as above, the contribution of locus mutation (point mutation and intralocus recombination) would be negligible in most theoretical plant and animal breeding studies."

If, then, it is satisfactory to regard the entire functional region as the basic unit of inheritance, the problem of position effects disappears. Such a solution seems appropriate for short-term selection theory. However, it might not be completely satisfactory for a theory pertaining to selection sustained for a very long time. In this case, it may be best to consider the mutational site as the basic hereditary unit, and hence the disturbance due to position effects should be examined.

Assuming, then, that the basic hereditary unit is the muton, the following argument considers the relative frequency, and hence the importance, of the position-effect phenomenon as it occurs among all possible pairwise combinations of mutons. It is assumed that the genotypic variability associated with the given quantitative variable is controlled by mutations, each of small effect at many mutons which are scattered at random over the chromosome complement.

The argument is: (i) the position-effect phenomenon is generated only by mutons in the same functional region and not by mutons in different regions, and (ii) in general, as the number of active regions increases, the frequency of pairwise combinations of mutational sites in different functional regions increases relative to the frequency of pairwise combinations of sites in the same functional region. Hence, when considering all possible pairs of mutons, the phenomenon becomes increasingly rare as the number of active functional regions increases.

This argument can be set out more rigorously as follows: Let there be n mutational sites (mutons) in each of N functional regions. Thus, there are a total of Nn sites. Of the total number of pairwise combinations $\binom{Nn}{2}$, there are $N\binom{n}{2}$ combinations of mutons in which both members of the pair are in the same functional region and hence may give rise to position effects. The remaining combinations, which number $\binom{N}{2}n^2$, have one muton in one functional region and the other in a different region. Hence they cannot give rise to positional effects. The relative proportion of pairs of sites in which position effects cannot occur is

$$\frac{\binom{N}{2}n^2}{\binom{Nn}{2}} = \frac{n[1 - (1/N)]}{[n - (1/N)]}.$$

Therefore, as the number of functional regions becomes large this proportion approximates one, irrespective of the number of mutons per functional unit.

The conclusion is that, even if position effects are widespread, the disturbance they cause to selection theory is negligible if the assumption holds that the genetic

variability is generated by genetic alternatives of small effect at many mutons (i.e. by genes of small effect at many loci).

Consider now the second phenomenon in which the individual is evaluated by its inbred progeny. In this case, the configuration effects are due to linkage and are not confined only to genes in close proximity but to all linked genes which do not show independent segregation.

Since these effects are directly a function of recombination values, the argument pertaining to the estimation of the average recombination value, as given by Griffing (1960b), is appropriate. It was shown that a fairly accurate estimate of the recombination value averaged over all possible pairs of active loci may be obtained from the formula

$$\bar{y} = (r-1)/2r,$$

where r = recombination index (Darlington 1958), i.e. the sum of the haploid number of chromosomes and the average number of chiasmata per cell.

This formula implies, roughly, that, of all pairwise combinations of loci, the relative proportion which segregates independently is $(r-1)/r$. Hence, as the recombination index increases, this value rapidly approximates one.

Again, this argument is subject to various assumptions and approximations, but nevertheless, it appears that if the haploid chromosome number is five or more (i.e. $r > 10$, because at least one chiasma per bivalent is obligatory), the disturbance due to linkage is probably not great. However, there are certainly instances in which configuration effects cannot be completely ignored. These include (1) cases in which crossing over is greatly reduced or non-existent (as in male *Drosophila*) in an organism which has a low chromosome number, and (2) cases in which interest centres on the manipulation of the gene content in a small number of chromosome pairs.

Finally, it is necessary to point out just where the configuration effects cause a disturbance to the prediction theory, if, in fact, they are appreciable.

It was stated previously that to predict *permanent* gains from selection, it is necessary to estimate the additive genetic variance. Configuration effects may then lead to biased estimates of this variance component. However, the estimation of the additive genetic variance component is not necessary to predict the *immediate* gains from selection. It can be shown that for both configuration-effect phenomena this prediction may be made directly from certain covariances. Since there is no theoretical difficulty in estimating these covariances, irrespective of the presence or absence of configuration effects, there is no bias in the estimation of immediate gains from artificial selection (ignoring, of course, the effects of natural selection).

It is clear, however, that the immediate gains may not be entirely sustained on relaxation of selection, and it is the gains which are retained after relaxation that are termed, here, the permanent gains. It is the prediction of these gains that may be biased by the presence of the configuration effects.

V. ACKNOWLEDGMENT

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ABNORMAL INHERITANCE OF THE SEX-LINKED TABBY GENE

By B. M. KINDRED*

[Manuscript received March 16, 1961]

Summary

Females of unexpected phenotype occur occasionally in mouse stocks segregating for sex-linked genes. Data from breeding of such females and frequency of occurrence in several different stocks are given. A possible explanation is advanced to account for the discrepancies between genetical and histological observations.

I. INTRODUCTION

In mouse stocks segregating for sex-linked genes, unexpected females of homozygous or hemizygous appearance occasionally occur. Russell, Russell, and Gower (1959) have shown that these cannot be the result of (a) change in dominance, (b) high mutation rate, or (c) sex reversal. Welshons and Russell (1959) demonstrate that it is improbable that the cause is an XXY -chromosome constitution, leaving the possibility that these females lack all or part of an X -chromosome. McLaren (1960) confirms these results although her frequency of occurrence of abnormal females is much lower. She suggests that the XXY constitution is male but only one animal of this apparent genotype was found. She also points out there is evidence in human beings, the only other mammals for which data on unbalanced sex-chromosome constitutions are available, that XO is a sterile female and XXY a male-type intersex.

White (1960) considers it probable that the Y -chromosome carries male determining factors in all mammals and that the few species previously reported to have XO males are really the result of chromosome fusion.

II. MATERIALS AND METHODS

In this Laboratory several selection lines segregating for tabby, a sex-linked, semi-dominant mutant, are being maintained. Heterozygous tabby mice typically have a striped coat and the number of secondary vibrissae on the face is reduced from 19 to about 15 (range 10–19). In the homozygous and hemizygous condition the hair types are markedly abnormal and the coat appears silky, the number of secondary vibrissae is reduced to 9 (range 5–13), and there are other characteristics such as bare patches behind the ears, lack of hair on the tail, and narrow eyes.

The stocks have different backgrounds and have been kept for varying periods up to 5 years so that large numbers of tabby mice have been recorded. The mating system was such that no homozygous tabby females were bred. Nevertheless, females which resembled hemizygous males did occur.

Although the primary purpose of the stocks was not related to the problem of abnormal chromosome numbers, any unusual appearance was recorded. All mice

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were scored for secondary vibrissa number and this could be used as a check on general appearance. Females which resembled $Ta\cdot$ males had vibrissa scores in the range expected for $Ta\cdot$ males or $TaTa$ females. The phenotype of $Ta+$ animals can be considerably altered by selection and in some lines $Ta+$ females which are phenotypically indistinguishable from $++$ females are common. Therefore, although $++\text{♀} \times Ta\cdot\text{♂}$ crosses have produced apparently wild-type females, these have only been included from lines where $Ta+$ females can be distinguished consistently. In considering the frequency of occurrence in different stocks, only TaO females, i.e. females resembling homozygous females, have been included (the symbol " O " represents the absence of an X -chromosome or the occurrence of an abnormal X -chromosome, contrasting with the " \cdot " symbol which represents the Y -chromosome).

TABLE 1
FREQUENCIES OF NORMAL AND ABERRANT TYPES OF MICE IN DIFFERENT GROUPS OF STOCKS

Stock	Mating	Progeny						
		$Ta+$	$++$	$Ta\cdot$	$+\cdot$	TaO	$+O$	$Ta+\cdot$
Group 1	$++ \times Ta\cdot$	588	—	—	534	7	—	1
	$Ta+ \times +\cdot$	484	477	424	442	19	—	—
Group 2	$++ \times Ta\cdot$	200	—	—	229	2	—	—
	$Ta+ \times +\cdot$	173	174	164	179	2	—	—
Group 3	$++ \times Ta\cdot$	1809	—	—	1861	2	2	—
	$Ta+ \times +\cdot$	1455	1442	1523	1538	5	—	1
Group 4	$++ \times Ta\cdot$	645	—	—	642	—	—	—
	$Ta+ \times +\cdot$	347	311	323	312	—	—	—

III. RESULTS AND DISCUSSION

Table 1 gives the frequencies of normal and aberrant types in different groups of stocks. There are 15 stocks altogether but these fall naturally in origin and frequency of TaO females into four different groups. Group 1 stocks are those in which the tabby gene was backcrossed into inbred strains A, CBA, C57, DBA, and 101. All showed a high frequency of TaO females. Group 2 are selection lines based on a cross between CBA and 101. Group 3 are selection lines based on CBA, 101, and two non-inbred stocks which contained the tabby gene. This group includes the only lines in which $+O$ individuals could be identified with certainty. Group 4 are selection lines based on a large randomly bred stock. The correlation between the degree of inbreeding in the stocks and the frequency of XO females is very marked and is quite sufficient to explain the differences found by Russell, Russell, and Gower (1959), whose *scurfy* stock was inbred to allow ovarian transplants, and by McLaren (1960), whose stocks appear similar to our type 3.

A few *TaO* females have been mated; the results are given in Table 2 and confirm the results of Welshons and Russell (1959). If the aberrant females had been *TaTa*· when mated to a $+ \cdot$ the classes of progeny produced would be: *Ta+*, *Ta*·, *Ta+·*, *Ta*··, or more rarely *TaTa+*, *TaTa*·, $+ \cdot$, ··. The only one of these genotypes which could possibly appear as a wild-type female is the *Ta+·*. As the apparently wild-type females did not transmit the *Ta* gene (no tabby mice were found in 29 progeny of two females) this hypothesis is untenable.

These data also show that in our stocks the egg lacking the *Ta* locus has a much lower viability than that found by Welshons and Russell. It is clear that the proportion of "wild-type" females produced by different *TaO* mothers varies (Table 2). This is also indicated in the data of Welshons and Russell (1959) where larger

TABLE 2
PROGENY OF *TaO* FEMALE MICE AFTER MATING WITH NORMAL MALES

Mating	Progeny			
	<i>Ta+</i>	<i>+O</i>	<i>Ta</i> ·	$+ \cdot$
<i>TaO</i> × $+ \cdot$	15	—	10	—
<i>TaO</i> × $+ \cdot$	6	—	8	—
<i>TaO</i> × $+ \cdot$	3	5	3	—

numbers of *TaO* females were mated although not many progeny were produced from each mating. This could be easily explained if "O" represents not lack of a whole chromosome but a deficiency, in which case it is only to be expected that a long deficiency would be less viable in an egg than a shorter deficiency.

Ohno, Kaplan, and Kinoshita (1959) have found with inbred strains no evidence of production of sperm either lacking *X*- and *Y*-chromosomes or possessing both *X*- and *Y*-chromosomes. They believe the occurrence of *XO* females is due to loss of an *X*-chromosome in early division. The data of Welshons and Russell and the breeding data given here show that *O* eggs can be functional although the viability varies. If *O* sperm do not occur there should be more *TaO* females from $+ + \times Ta \cdot$ matings than from *Ta+ × +·* matings. In the former the *Ta* chromosome comes from the male and a loss or deletion of either of the $+$ chromosomes of the female can survive in the egg to produce a *TaO* female. These can also be produced by an error in cleavage. Any *TaO* females from *Ta+ × +·* matings must have the *Ta* chromosome from the female parent and, if *O* sperm do not occur, must all represent the loss of an *X*-chromosome in early cleavage.

The data show 26 *TaO* females from *Ta+ × +·* matings and only 11 from $+ + \times Ta \cdot$ matings. Either *O* sperm do occur or errors in cleavage are much more frequent when the mother is *Ta+·*. In view of the difference in viability of *O* eggs

from different mothers it seems more likely that the former is correct but that the *O* sperm had only part of a chromosome missing and were not detected by the histological methods of Ohno, Kaplan, and Kinoshita (1959).

The striped male found by McLaren (1960) died before reaching maturity but she suggests that it was *Ta*+ . Two more males of this type have appeared in our experiments; both were strong and healthy but although each was mated to several females known to be fertile, no offspring were produced. It was therefore not possible to confirm that these animals were *XXY*.

If the *TaO* females have a deletion of the *Ta* locus rather than lack of a whole chromosome there must be an exceptionally high rate of spontaneous deficiencies; particularly as this phenomenon has also been observed with *bent tail* and *scurfy*. However, until this possibility can be eliminated the grounds for assuming that the *XO* chromosome constitution is female are uncertain.

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ADDENDUM

Cytological examination by D. L. Hayman (University of Adelaide) shows that one male, presumed *XXY*, has $2n = 41$ chromosomes, and one aberrant female has $2n = 39$. She consequently lacks a whole chromosome and not part as suggested.

THE ACTION OF UREA ON DIAPAUSE IN EGGS OF *ACHETA* *COMMODUS* (WALK.) (ORTHOPTERA: GRYLLIDAE)

By T. W. HOGAN*

[Manuscript received March 1, 1961]

Summary

Urea, at concentrations of 0.04–0.08M, was found to prevent the onset of diapause in eggs of *Acheta commodus* (Walk.) when applied during the prediapause stages of development.

Higher concentrations, up to 0.24M, were required to terminate diapause in eggs that had already entered diapause. The rate of termination declined as the concentration of urea was reduced; the lowest concentration causing appreciable increase in the rate of termination was about 0.006M. The upper limit to the effective strength was imposed by toxic effects of urea.

An additional experiment is described in which interrelation between the effects of exposure to low temperature and the application of urea on the rate of elimination of diapause was measured.

I. INTRODUCTION

The rate of termination of diapause in eggs of the field cricket *Acheta commodus* (Walk.) has a negative temperature coefficient within the range -16.5°C to $+5^{\circ}\text{C}$ (Hogan 1960b). Similar responses to temperature have been obtained in eggs of *Leptohylemyia coarctata* Fall. (Way 1960).

A negative temperature coefficient for a steric change in a protein, viz. the initial stages of the denaturation of β -lactoglobulin by urea, has been demonstrated by Jacobsen and Christensen (1948).

This was of interest because certain of the characteristics of diapause, including its termination by physical agencies, such as by abrasion, would be most readily explained in terms of a steric change. In view of this and since a negative temperature coefficient for a biological reaction is a rare phenomenon, the possibility that diapause in *Acheta* is terminated by a similar process to the denaturation of β -lactoglobulin was investigated.

In order to incorporate chemicals into the eggs, use was made of the characteristics of orthopteran eggs whereby at one stage of development a considerable quantity of water is absorbed over a relatively short period. In *Acheta* an amount almost equal to the original weight of the egg is absorbed during the fourth and fifth days at 27°C (Browning 1953), commencing almost coincidentally with the onset of diapause (Hogan 1960a).

In subsequent experiments it was found that urea could eliminate diapause, whether applied before or after water uptake, suggesting that the action might be on the egg cuticle. Tests with radioactive isotopes, however, have shown that absorp-

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tion of the urea into the egg takes place. These observations will be reported in a separate paper.

II. METHODS AND MATERIALS

The eggs were obtained from cultures of field crickets maintained in the laboratory. To guard against the selection of strains the cultures were only one generation removed from crickets collected in the field. Conditions for oviposition and for incubation of the eggs were as described in a previous paper (Hogan 1960a).

The main criterion used to measure the effect of treatments on diapause was the percentage of eggs that hatched without evidence of diapause. The median period required by diapause-free eggs to complete their development at 27°C is 15 days (Hogan, unpublished data).^{*} In these experiments eggs that hatched within 3 days of the median period were considered to be diapause-free. However, when the effect of treatment was not very marked the measure used was the period taken for 50% of the eggs to hatch. This is termed the median effective duration of exposure (M.E.D.E.). An alternative measure in such cases was the percentage hatching in successive weeks after the hatching of diapause-free eggs.

The urea solutions were prepared from analytical grade reagents.

In each experiment the eggs were placed on disks of blotting-paper saturated with a solution of the chemical under test and held in sealed plastic tubes at 27°C. Three replicates of 25 eggs per tube were used in each treatment. The control treatment was the same except that distilled water replaced the chemical solution and the percentage of eggs in this treatment that hatched without evidence of delay was taken to be a measure of the strength of diapause in the eggs. Actually this measures the strength of the tendency to enter diapause. These two factors seem to have a positive relationship, but the exact correlation has not been measured. In Section IV the need for a more satisfactory criterion of strength of diapause is indicated.

The term "elimination of diapause" as distinct from "termination of diapause" has been used where the eggs developed, after treatment, without evidence of diapause although they may have experienced one of brief duration.

III. RESULTS

(a) *Effect of Denaturants*

Solutions of urea were applied to prediapause eggs by the method described in Section II. This proved highly toxic at the concentrations normally used for the denaturation of protein (4–7M). This toxicity, however, was assumed to be an indication of absorption of the urea by the eggs and further tests were carried out at less toxic levels. These were in several series, and in the series at the lowest concentrations, in which only slight toxic effects were observed, a definite effect on the elimination of diapause was obtained at 0.08M (Table 1). The experiments were, therefore, continued with urea and are described in the later sections of this paper. Tests were also made on other denaturants. L-Guanidine hydrochloride at concentrations of

^{*} Browning quotes 13 days for the mean duration at 26.8°C but this was after 30 days at 12.8°C during which embryogenesis proceeds slowly.

0.01–2M had no visible effect. Phenylthiourea (0.01M) was highly toxic whilst thiourea at concentrations of 0.01–1M was also highly toxic but with some indication of an effect on diapause in surviving eggs at the lower concentrations.

It was concluded that the lack of toxicity of 2M guanidine probably meant that it was not absorbed into the eggs. Any effects on diapause that phenylthiourea might have had were obscured by its high toxicity at the concentration tested. The rate of elimination of diapause in the eggs surviving concentrations of 0.01–0.04M thiourea was slightly higher than for the control. Further investigation of both of these materials would seem to be worth while.

TABLE 1
PERCENTAGE OF VIABLE EGGS THAT DEVELOPED WITHOUT EVIDENCE OF DIAPAUSE AFTER EXPOSURE, DURING THE PREDIAPAUSE STAGES, TO SOLUTIONS OF UREA AT THE CONCENTRATIONS INDICATED

Urea Concn. (M)	Arcsin ($H^{\frac{1}{2}}$)* (degrees)	Retransformed Percentage Hatch	Mortality (%)
0	12.5	4.7	5
0.02	13.2	5.2	0
0.04	16.5	8.0	4
0.08	41.2	43.4	32

* H = percentage hatch; difference for significance at the 5% level = 13.1, at the 1% level = 19.0.

(b) *Optimum Concentration of Urea*

In the experiments described in Section III (a) a marked increase in the percentage of eggs developing without evidence of diapause was obtained with a 0.08M solution of urea.

In order to determine the optimum concentration for this effect urea solutions ranging from 0.01 to 0.16M (see Table 2) were applied to eggs not more than 24 hr old, and kept in contact with them throughout the incubation period at 27°C. The effect of the treatments was measured by the percentage hatching in a period corresponding to diapause-free hatching, and by a further count 2 weeks later.

Although the maximum response is estimated to be at 0.03M (Table 2) it is evident, from a consideration of differences for significance, that the maximum is not sharply defined in the range 0.02–0.04M. When further counts were made after a total period of 31 days the percentage hatches at 0.02, 0.03, and 0.04M were closely similar (59.6, 64.4, and 65.7), and substantially higher than the percentage hatch at 0.08M (18).

A series of low concentrations was tested separately (Table 2) and, when measured in terms of the period of exposure required for a 50% hatch, demonstrated that, for these eggs, the threshold was about 0.006M; and that the rate of elimination increased with increase in the concentration of urea.

(c) *Timing and Dosage of Urea*

In the previous experiments the eggs were treated with the urea solutions shortly after oviposition and remained there until the completion of hatching. The assumption was made that the urea was incorporated into the eggs during the process of water uptake, but this had not been proved.

TABLE 2
EFFECT OF DIFFERENT CONCENTRATIONS OF UREA ON THE RATE OF ELIMINATION OF DIAPAUSE
WHEN INCUBATED AT 27°C

Rate Measured by Diapause-free Hatching				Rate Measured by Period Taken for 50% Hatch	
Urea Concn. (M)	Arcsin ($H^{\frac{1}{2}}$)* (degrees)	Retransformed Percentage Hatch after 17 Days	Mortality (%)	Urea Concn. (M)	Median Effective Duration of Exposure (days)
0	13.7	5.6	3	0	54
0.004	20.4	12.2	3	0.006	47
0.02	27.7	21.6	7	0.01	40
0.03	39.6	40.6	3	0.02	35
0.04	25.3	18.3	0	0.04	23
0.08	19.9	11.6	4		
0.16	16.1	7.7	36		

* H = percentage hatch; difference for significance at the 5% level = 21.1, at the 1% level = 29.4.

In order to determine at what stage of development the action took place, the contact of the eggs with the urea solution was restricted to particular stages of development.

(i) *Effect of Exposure to Urea during the Period of Water Uptake.*—These effects were compared with those of exposure to urea during the whole of prediapause development. The results (Table 3) show that the longer the period of exposure during prediapause development the more marked is the effect. This suggests that the total period of exposure is important rather than just the period of water uptake. The lower percentage hatching of the eggs in contact with urea throughout the incubation period possibly resulted from some overdosing, sufficiently unfavourable to reduce the rate of development. It was not because of lethal effects.

TABLE 3
EFFECTS OF UREA WHEN APPLIED AT DIFFERENT STAGES OF DEVELOPMENT OF THE EGG

Day of Treatment	Urea (0.03M) Applied during Prediapauses Stages				Urea (0.04-0.24M) Applied during Diapause				
	Arcsin ($H\frac{1}{2}$)* (degrees)	Retransformed Percentage Hatch	Mortality (%)		Urea Concn. (M)	Arcsin ($H\frac{1}{2}$)† (degrees)	Retransformed Percentage Hatch after 17 Days	Mortality (%)	Hatch‡ after 30 Days (%)
No urea	14.4	6.2	0		0	5.5	1.0	1	11
1st-5th	26.6	20.0	0		0.04	11.8	4.2	4	43
1st-6th	43.8	47.9	0		0.08	31.2	26.8	8	87
4th and 5th	21.5	13.4	3		0.16	61.2	76.8	12	86
4th, 5th, and 6th	26.4	19.8	0		0.24	48.6	56.3	53	47
Continuous	21.9	13.9	3						

* H — percentage hatch; difference for significance at the 5% level = 12.7, at the 1% level = 17.3.

† Difference for significance at the 5% level = 28.1, at the 1% level = 39.9.

‡ Values not transformed.

(ii) *Effect of Exposure to Urea after Water Uptake*.—As the result of evidence obtained in several pilot tests, an experiment was set up to determine the effects of the application of urea after water uptake had been completed and the eggs had entered diapause. For this purpose the eggs were held at 23°C for 14 days before treatment, and higher concentrations of urea, from 0.04 to 0.24M, were used. The results (Table 3) reveal that these concentrations of urea could cause the termination of diapause. The most effective concentration in this case was 0.16; beyond this, at 0.24M, the mortality was high (53%).

TABLE 4

INFLUENCE OF UREA ON THE RATE OF ELIMINATION OF DIAPAUSE IN EGGS PREVIOUSLY EXPOSED TO LOW TEMPERATURE

Urea Concn. (M)	No. of Days at 12°C	Arcsin ($H^{\frac{1}{2}}$)* (degrees)	Retransformed Percentage Hatch	Urea Concn. (M)	No. of Days at 12°C	Arcsin ($H^{\frac{1}{2}}$)* (degrees)	Retransformed Percentage Hatch
0.04	0	38.9	39.4	0	0	23.7	16.2
0.04	5	60.0	75.0	0	5	41.1	43.2
0.04	14	24.1	16.7	0	14	57.0	70.3

* H = percentage hatch; difference for significance at the 5% level = 23.6, at the 1% level = 33.4.

Browning and Forrest (1960) have shown that in non-diapause eggs exchange of water occurs at all stages of development, but this would not be expected to apply to diapause eggs. It might be supposed that the urea was acting only on the cuticular coverings causing a change in their permeability, but an investigation with radioactive isotopes has shown that the urea is, in fact, absorbed into diapause eggs.

(d) Combined Effect of Low Temperature and Urea

Since urea can cause the elimination, or termination, of diapause in *Acheta*, the possibility that it is the agent under natural conditions must be considered. It is true that the usual end-product of nitrogen metabolism in insects is uric acid, but the presence of urea has been recorded in a number of insect species (see, for example, Bheemeswar 1958). Hence the synthesis of urea, and its accumulation until an effective concentration is reached, could be the mechanism by which diapause in *Acheta* is eliminated when eggs are exposed to temperatures of about 10°C.

If urea is indeed synthesized at low temperatures then the effectiveness of applied urea should increase after the eggs have been exposed to such, provided the optimum dosage of urea is not exceeded. Beyond this latter level unfavourable effects would be expected to occur, corresponding to the responses obtained when concentrations of urea beyond the optimum are applied to the eggs.

An experiment was therefore carried out in which eggs, not more than 16 hr old, were held at 12°C for 0, 5, and 14 days and then treated with 0.04M urea during

incubation at 27°C. Another group, from the same batch of eggs, was given the same treatments but without the addition of urea.

Table 4 shows that after an exposure of 5 days at 12°C followed by treatment with 0.04M urea, the rate of elimination of diapause was higher than after either of these treatments alone, but after 14 days at low temperature the rate was substantially lower.

If it be assumed that the optimum concentration for these eggs was higher than 0.04M, then the data are consistent with a hypothesis that urea is synthesized during exposure to low temperature: but the results do not exclude other interpretations.

IV. DISCUSSION

The foregoing experiments have demonstrated that urea, a well-known denaturant, has the capacity to terminate diapause from eggs of *A. commodus*. The feasibility of the action being denaturant is supported by some of the characteristics of diapause in other species, e.g. the response to physical agencies, the rapidity of such responses, and the reversibility of diapause recorded by Salt (1947). The fact that a negative temperature coefficient has been found for a denaturant action and also exists for the termination of diapause in *Acheta* seems significant.

Against this the chief objection appears to be that the negative temperature coefficient for denaturation in urea, "seems to be rather special to β -lactoglobulin and not to be a general property of the proteins" (Dr. M. Ottesen, personal communication). Another objection is the low concentration of urea found to be effective. However, denaturation, although an extremely complex process, includes comparatively simple steric changes such as the breaking of hydrogen bonds. Klotz (1958), who has reviewed one such change, viz. the hydration of protein molecules, states that the breakdown of the water lattice by urea reveals itself (among other ways) by unmasking of previously inactive groups. As far as unmasking is concerned, "even a 1M solution produces a visible effect" (Klotz, personal communication). Jensen (1955) remarks that relatively mild conditions may affect sulphhydryl-disulphide interchange—again affecting the reactivity of proteins. Moreover, although concentrations of urea as low as those used in the experiments on *Acheta* have not been recorded as causing steric changes, the long periods of exposure at low concentrations may have effects not observable with *in vitro* chemical reactions.

Urea is effective in increasing the rate of termination of diapause in *Acheta* when applied at concentrations within the range 0.006–0.16M. (The concentration at the site of action is not known.) The upper limit of this range is set by toxic effects, the onset of which overlaps those concentrations most effective in terminating diapause. This is reminiscent of the effect of subzero temperatures (Hogan 1960*b*).

The optimum concentration of urea, measured by diapause-free hatching was 0.08M in one test (see Table 1) and 0.03M in another (Table 2). This discrepancy is not due to a difference in the onset of mortality which was the same in both experiments. Nor does it appear to be linked with the intensity of diapause as measured by the control treatments, as this, too, was very similar. A number of possibilities exist but one question that arises is whether the method of measuring the intensity

of diapause is a satisfactory one. Further experiments will be necessary to elucidate this point.

Apart from the nature of the action of urea there is the question as to whether urea could be the agent causing the termination of diapause under natural conditions. The effectiveness of exposure to temperature of about 10°C for 1-3 months could be readily explained in terms of the slow synthesis of urea during this period and its accumulation until an effective concentration is reached. Perhaps at higher temperatures a balance between breakdown rate and synthesis would account for the decrease in effect. Schneiderman (1956) has suggested that such a process (in relation to an unknown agent) could be the mechanism of termination in postembryonic diapause.

V. ACKNOWLEDGMENTS

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THE FATE OF INTRAVENOUS DOSES OF FREE AND PLASMA PROTEIN-BOUND [^{35}S]CYSTINE IN THE SHEEP

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Summary

When a tracer quantity of L- or DL-[^{35}S]cystine was mixed with sheep blood plasma about two-thirds of the radioactivity was bound to the proteins after 4 hr at 39°C, probably as half-cystine residues in disulphide linkage. The reversibility of the binding was demonstrated.

Intravenous doses of L-[^{35}S]cystine, both as the free amino acid and as half-cystine residues bound by disulphide bonds to plasma proteins *in vitro*, were given to sheep, and the fate of the ^{35}S studied. The results suggested that at least some of the cystine not in peptide linkage is bound to the plasma proteins *in vivo* and that there is a continuous exchange between the free and bound forms with a half-time of about 1 hr. It was not possible to decide from these experiments whether the free or the disulphide-bound cystine play any special role in keratinization.

On the average, about 30% of an intravenous dose of L-[^{35}S]cystine appeared in the wool grown during the 28 days after the dose, whether the cystine was given in the free or the disulphide-bound form or whether the specific activity was lowered by the dilution of the dose with as much as 2 g of "carrier" L-cystine. The significance of these results is discussed.

I. INTRODUCTION

During a previous study of the mechanism of incorporation of [^{35}S]cystine into wool (Downes 1961) it was found that a large proportion of the radioactivity became bound to the plasma proteins *in vitro*. Cystine added to plasma evidently equilibrates rapidly with free cysteine and cystine (cyst(e)ine) and with half-cystine residues held by disulphide bonds by reaction with the -SH groups of the protein molecules. The bound cystine is not removed by precipitation of the proteins with trichloroacetic acid but is removed by treatment with reducing agents such as mercaptoethanol (Lee *et al.* 1951) or NaHSO_3 (Downes 1961). If there is significant binding of cyst(e)ine to plasma proteins *in vivo* it would be important to know if the bound form plays any special role in the synthesis of wool keratin. For example it is possible that cyst(e)ine has to be carried in the bound form to the skin or the wool follicles to be extracted by disulphide interchange.

Further studies on the binding of cystine by plasma proteins *in vitro* have now been made. Other experiments described here were carried out to see if the binding of cystine by the plasma proteins occurs *in vivo* and to study the fate of the bound cystine in the sheep. The efficiency of incorporation into wool of intravenous doses of L-[^{35}S]cystine diluted with various amounts of non-radioactive L-cystine was also measured.

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II. MATERIALS AND METHODS

The doses of L-[^{35}S]cystine (from the Abbott Laboratories, Chicago, U.S.A.) and of DL-[^{35}S]cystine (from the Radiochemical Centre, Amersham, England) were prepared and the radiochemical purity checked as described previously (Downes and Lyne 1961).

The experimental animals (three Corriedale wethers and one ewe) were kept in metabolism cages in an animal house and were fed a constant daily ration of 800–1000 g of equal parts of lucerne and wheaten chaff.

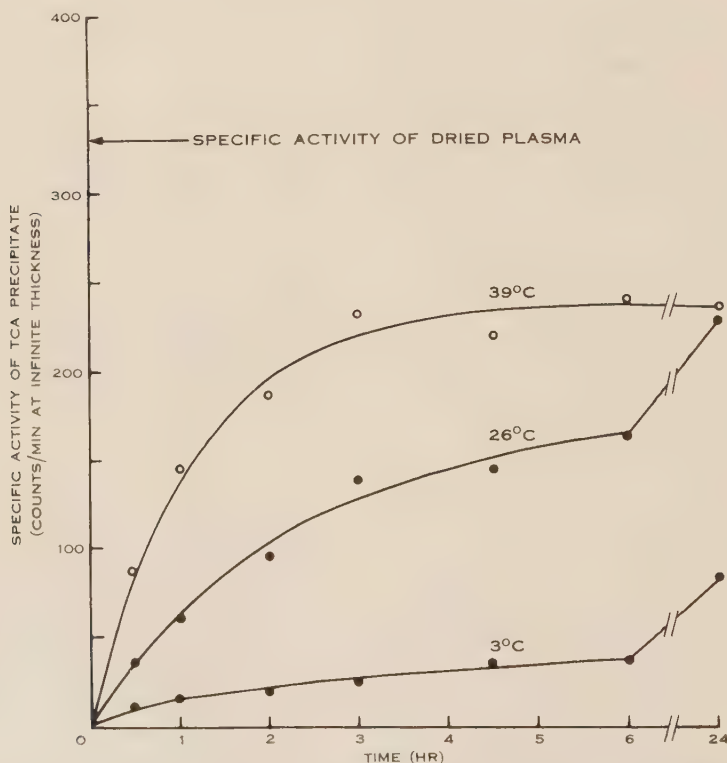


Fig. 1.—Rate of binding of [^{35}S]cystine by plasma proteins at different temperatures. To each of six 10-ml samples of plasma freshly obtained from a sheep was added 0.1 ml of aqueous L-[^{35}S]cystine (16 μg ; 0.31 μc) as the hydrochloride. Two of the mixtures were kept at 39°C, two at 26°C, and two at 3°C. Samples were removed at intervals and the proteins precipitated with TCA. The results are the means of each pair of duplicates. The specific activity of the dried plasma is also shown to indicate the degree of binding.

The methods of radioassay and of blood analysis were those described by Downes (1961) except that the plasma proteins were washed with acetone, instead of with ethanol and ether, after precipitation with trichloroacetic acid (TCA). The ^{35}S present in TCA precipitates of plasma which had been pretreated with NaHSO_3 was assumed to be "peptide-bound ^{35}S ", that is with the cystine present in peptide linkage. The extra ^{35}S in TCA precipitates of untreated plasma is

referred to as "disulphide-bound ^{35}S ", presumably present as half-cystine residues bound by disulphide bonds.

The wool samples were cleaned by washing with ether, ethanol, and water, and were heated at 110°C for 18 hr. Samples were analysed for sulphur by the method of Myers (1959). Other samples were oxidized by the method of Myers (1959) and counted as benzidine sulphate at "infinite" thickness. All measurements of ^{35}S were referred to the counting rates of standards prepared from the labelled cystine.

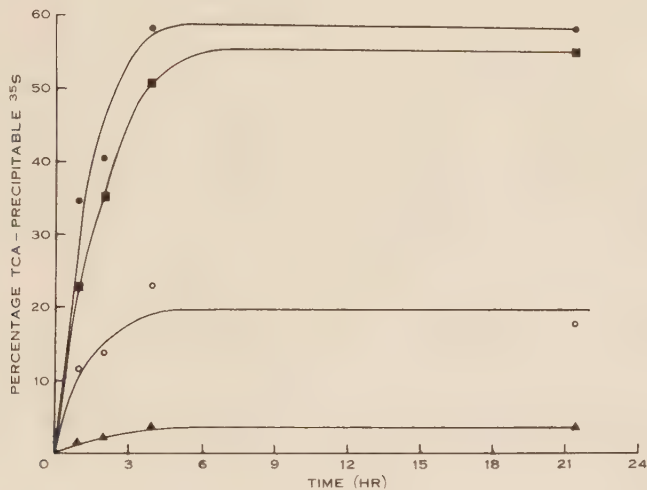


Fig. 2.—Effect of carrier cystine on the binding of $[^{35}\text{S}]$ cystine by plasma proteins. To each of four 8-ml portions of sheep plasma at 39°C was added 0.2 ml of a solution of DL- $[^{35}\text{S}]$ cystine hydrochloride. Samples were removed at 1, 2, 4, and 21.5 hr and the proteins precipitated with TCA. The amounts of cystine used and their specific activities were:

- | | |
|--------------------------------|-------------------------------|
| ● 0.040 mg, 0.68 μc | ○ 1.51 mg, 0.68 μc |
| ■ 0.160 mg, 2.70 μc | ▲ 11.1 mg, 0.68 μc |

III. EXPERIMENTAL RESULTS

(a) Binding of Cystine by Plasma Proteins *in vitro*

Solutions of $[^{35}\text{S}]$ cystine hydrochloride were incubated at various temperatures with blood plasma freshly obtained from sheep. This treatment altered the pH of the plasma by no more than 0.05 units. The binding was followed by removing samples (1 ml) at intervals and precipitating the proteins with TCA (4 ml, 10% w/v). The precipitates were washed three times with TCA (5% w/v) and several times with acetone, dried, and their specific activities measured.

The results in Figures 1 and 2 show that ^{35}S rapidly became bound to the plasma proteins *in vitro*. At 39°C an equilibrium appeared to be established in about 4 hr but at lower temperatures the rate of binding was slower. From Figure 2 it may be seen that the larger the amount of cystine added the smaller was the percentage

of bound ^{35}S . With very small amounts of added cystine (20 and 5 μg per ml plasma) practically the same percentage was obtained, showing that the amount of free plus bound cystine in the plasma must be much larger than 20 $\mu\text{g}/\text{ml}$. As shown in Figure 3 the further addition of a relatively large mass of non-radioactive cystine removed a large proportion of the bound ^{35}S .

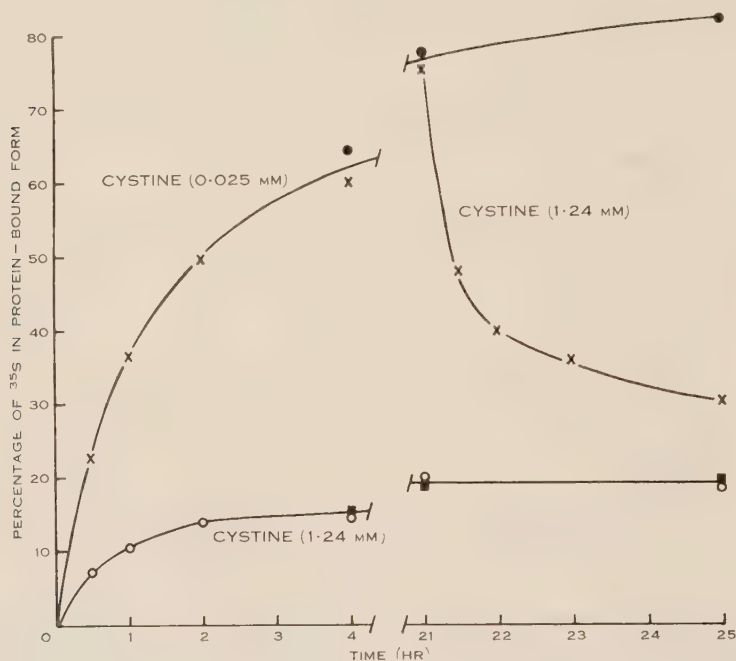


Fig. 3.—Demonstration of the reversibility of binding of L- ^{35}S cystine by sheep plasma proteins. Four samples of plasma were heated at 39°C with L- ^{35}S cystine and 1-ml portions removed at intervals for the measurement of bound ^{35}S . Two of the solutions contained added cystine at a concentration of 1.24 mM; in the other two the concentration was 0.025 mM. After 21 hr one of the latter solutions was also made 1.24 mM with respect to added cystine and the sampling continued for a further 4 hr.

From Table 1 it may be seen that the bound ^{35}S was not removed by dialysis overnight against water and that the same results were obtained when picric acid was used as the precipitant instead of TCA. Other experiments, in which the proteins were similarly labelled, showed that 30–70% of the bound radioactivity was lost when the proteins were coagulated by boiling the solutions for a few minutes.

At equilibrium about 60–80% of the radioactivity was precipitable with TCA when tracer amounts of cystine were added. DL- ^{35}S cystine was similarly mixed with fresh plasma (about 1 μg cystine per ml plasma) from several other species. In each case curves similar to those in Figures 1 and 2 were obtained. The species used and the percentage of ^{35}S precipitable with TCA after 24 hr at 20°C were: man 11, cow 51, domestic fowl 6, rat 78, rabbit 71, and brush-tailed possum 43.

(b) Binding of Cystine by Plasma Proteins in vivo

L-[^{35}S]cystine ($356\ \mu\text{C}$; $6.7\ \text{mg}$) was injected intravenously into a Corriedale ewe. Blood samples were taken at intervals, cooled immediately to below 5°C , and centrifuged at $2700\ g$ for 30 min at 5°C . The plasma was then analysed as soon as possible for total ^{35}S , peptide-bound ^{35}S , disulphide-bound ^{35}S , free cystine ^{35}S , and sulphate ^{35}S . The proteins were precipitated no later than 1 hr after the blood samples were taken.

The changes which occurred in the distribution of ^{35}S in the blood plasma during the first 7 hr are shown in Figure 4. Since the plasma samples were processed

TABLE 1

SPECIFIC ACTIVITIES OF PROTEIN SAMPLES OBTAINED IN THREE WAYS FROM PLASMA-[^{35}S]CYSTINE MIXTURES

Sheep plasma (5 ml) and L-[^{35}S]cystine ($0.19\ \mu\text{C}$; $16\ \mu\text{g}$ in $0.5\ \text{ml}$ 0.9% NaCl) were mixed and heated at 39°C for 6 hr. Duplicate samples of the proteins were then obtained by the methods listed and their specific activities measured. The counting rate of the dried plasma is included to indicate approximately the maximum specific activity possible

Sample	Specific Activity (counts/min at infinite thickness)	Sample	Specific Activity (counts/min at infinite thickness)
Dried plasma	333 321	TCA precipitate of sample dialysed overnight against running tap water	240 223
TCA precipitate	222 242	Picric acid precipitate*	252 228

* Obtained by method of Stein and Moore (1954).

immediately and kept cold (5°C) throughout, it was estimated from Figure 1 that no more than 10% of the free cystine- ^{35}S in each sample could have become bound to the proteins during the processing. Nevertheless the amount of non-peptide radioactivity in the bound form increased to about 70% during the first hours after the injection. The absolute amount of bound ^{35}S was maximal about 15 min after the injection and then declined approximately exponentially with a half-time of about 2 hr.

Similar results were obtained when this experiment was repeated with another sheep. On this occasion some of the TCA precipitate (281 mg) of plasma from a blood sample taken 1 hr after the injection was oxidized with performic acid, hydrolysed, and the cysteic acid isolated from the hydrolysate as described previously (Downes 1961). The TCA precipitate contained 55% of its radioactivity as peptide-bound ^{35}S and the cysteic acid recovered accounted for at least 80% of the initial ^{35}S . Since some losses undoubtedly occurred during the above procedure it is evident that the bulk of the initial ^{35}S was present as half-cystine residues.

(c) *Efficiency of Incorporation of L-[³⁵S]Cystine into Wool*

Three Corriedale wethers were given a series of intravenous injections of L-[³⁵S]cystine, as the free amino acid or in the partly disulphide-bound form.

A typical dose of plasma-containing disulphide-bound L-[³⁵S]cystine was prepared as follows. About 100 ml blood was collected using heparin (1 ml, 1% w/v) as the anticoagulant, and immediately centrifuged for 40 min at 600 *g* and 5°C. The bulk of the plasma was transferred to a flask containing L-[³⁵S]cystine (28.2 μ c; 540 μ g cystine as the hydrochloride in 0.126 g aqueous solution). The mixture was kept for 8 days at 5°C (to avoid growth of bacteria in case of accidental contamination) and then injected intravenously into the same sheep. In the experiments with sheep MA51 and MA52 appropriate precautions were taken to keep the plasma and the L-[³⁵S]cystine sterile throughout. Just before each injection a sample of the labelled plasma was taken for the estimation of the percentage of disulphide-bound ³⁵S. In each case this was about 60% of the total ³⁵S. Samples of the mixture injected into MA51 and MA52 were also taken for bacteriological examination and found to be sterile. Two of the sheep (MA50, MA52) received the labelled plasma first and a dose of free L-[³⁵S]cystine about 4 weeks later. This order was reversed in sheep MA51.

The importance of the specific activity of the doses of free cystine was determined by giving each of the three sheep another dose of free L-[³⁵S]cystine diluted with 0.10, 0.50, and 2.0 g respectively of carrier.

The fleece was clipped as completely as possible just before each injection. Two tattooed areas, one on each side of the sheep, were clipped weekly. The mass of clean dry wool and the specific activity of the benzidine sulphate derived from each sample were measured. Blood samples were taken during the 6 hr after each injection and the specific activity of samples of the dried plasma was measured.

The doses and amounts of ³⁵S incorporated into the wool are summarized in Table 2. To illustrate the reproducibility of the results and the method of calculation, the details of two of the experiments with sheep MA51 are given in Table 3. Corrections were made for the contribution to the total radioactivity from the previous doses using the data from two detailed specific activity *v.* time curves obtained previously. The largest correction was about 13% of the total ³⁵S in the fleece from the dose being studied.

In one comparison only (sheep MA51) the percentage of the dose incorporated into the wool was larger for the bound than for the free cystine. In each case the results for the free cystine were about the same even though the mass of cystine injected ranged from a fraction of a milligram to 2 g.

The initial rates of disappearance of the radioactivity from the circulating plasma after some of the doses of free and of disulphide-bound L-[³⁵S]cystine are shown in Figure 5. The results show that at least two-thirds of the free cystine disappeared from the blood stream in the first few minutes, irrespective of the mass of cystine injected. Thus, when the plasma-cystine mixtures were injected the free cystine was presumably removed in the first few minutes. Subsequently the rate of disappearance of the ³⁵S during the first 2 hr followed an exponential

function with a half-time of about 1 hr. This must approximately represent the disappearance of the disulphide-bound radioactivity since the amount of incorporation of cystine- ^{35}S by protein synthesis during the 2-hr period was estimated to be no more than 20%.

TABLE 2

EFFICIENCY OF INCORPORATION OF ^{35}S INTO THE WOOL OF SHEEP AFTER INTRAVENOUS INJECTIONS OF L- ^{35}S CYSTINE

Sheep No.	Dose			^{35}S in Fleece Grown during the First 4 Weeks after Each Dose (%)
	Form	Mass of Labelled Cystine (mg)	Amount of ^{35}S (μc)	
MA50	Cystine + plasma*	0.40	25.1	30
	Free cystine	0.64	40.3	28
	Free cystine	100	40.3	35
MA51	Free cystine	0.64	40.2	24
	Cystine + plasma*	0.32	20.0	37
	Free cystine	2000	40.3	24
MA52	Cystine + plasma*	0.43	27.3	28
	Free cystine	0.64	40.4	31
	Free cystine	500	40.2	30

* About 60% of the ^{35}S was in the form of half-cystine residues bound to the plasma proteins by disulphide bonds. See text for full description.

IV. DISCUSSION

The binding of cystine (or cysteine) and other compounds containing disulphide or sulphydryl groups to proteins is a well-known phenomenon which is especially important in the study of such compounds by the radioactive tracer technique (Lee *et al.* 1952; Tarver 1954; Samarina, Kritzman, and Konikova 1956; Eldjarn and Pihl 1956, 1957). Eagle, Oyama, and Piez (1960) attributed at least a part of the slow but continuing growth which was observed in mammalian cell cultures in a cyst(e)ine-free medium to the mobilization of bound cyst(e)ine. The marked stimulatory effect in this system of reduced inorganic sulphur compounds and of compounds such as D-cystine and glutathione was similarly attributed to the fact that they promote the release of the bound cystine residues from the serum proteins. They concluded that a reversible transfer of a half-cystine residue (CyS) from the cystine (CySSCy) to a protein thiol group (PrSH) occurred:



The present results (Figs. 1 and 2) agree with this and show that about two-thirds of a tracer amount of cystine is bound to the proteins of sheep plasma *in vitro* after

TABLE 3

SHOWING THE METHOD OF ESTIMATION OF THE PERCENTAGE OF DOSE OF ^{35}S IN THE FLEECE OF SHEEP MA51 AFTER TWO INTRAVENOUS INJECTIONS OF L- ^{35}S CYSTINE

Dose	Clipped* Area	Growth Period (days)	Mass of Clean Dry Wool (g)	Specific Activity† ($\mu\text{c/g}$)	Total ^{35}S in Sample (μc)	Total ^{35}S in Fleece (μc)	% Dose in Fleece
40.2 μc free cystine (0.64 mg) injected on day 0	I	0-7	3.50	0.053	0.186	9.49‡	24
		7-14	4.32	0.131	0.567		
		14-21	4.05	0.027	0.109		
		21-28	4.21	0.015	0.062		
	Totals		16.1		0.924		
	II	0-7	2.85	0.042	0.120	9.15	
		7-14	3.59	0.130	0.465		
		14-21	3.37	0.029	0.099		
		21-28	3.37	0.014	0.048		
	Totals		13.2		0.732		
	Whole fleece	0-28	165	0.0582 0.0589 0.0621 0.0586		9.81	
	Means			0.0595		9.48 (9.5)§	
20.0 μc cystine + plasma injected on day 28	I	28-35	4.73	0.0457	0.216	8.18	37
		35-42	4.39	0.0792	0.348		
		42-56	10.64	0.0157	0.167		
		56-63	4.96	0.0093	0.046		
	Totals		24.7		0.777		
	II	28-35	4.00	0.0438	0.175	7.84	
		35-42	3.57	0.0756	0.270		
		42-56	9.02	0.0172	0.155		
		56-63	4.75	0.0088	0.042		
	Totals		21.3		0.642		
	Whole fleece	28-63	260	0.0347 0.0310 0.0347 0.0296		8.45	
	Means			0.0325		8.16 (7.14)§	

* The whole fleece was clipped just before each injection. Two tattooed areas (I and II), one on each side of the sheep, were usually clipped weekly.

† The sulphur content of the wool (3.02, 3.04, 3.02, 3.09; mean 3.04%) was determined by the method of Myers (1959). Other samples of the wool were oxidized by the method of Myers (1959) and the specific activity of the sulphur, as benzidine sulphate, was measured. The specific activity of the wool was then calculated.

‡ i.e. $0.924 \times 165/16.1 = 9.49$.

§ Values in parenthesis are mean values corrected for previous doses.

4 hr at 39°C. About the same proportion was bound whether DL- or L-[³⁵S]cystine was used, confirming that D-cystine can take part in the exchange. The binding occurred to some extent in the plasma from all the species examined, showing that the reaction is not a property of sheep plasma proteins only.

Eagle, Oyama, and Piez (1960) found that -S-S- and -S- compounds, including cystine, failed to effect significant dissociation of the bound ³⁵S, whereas the results here (Fig. 3) showed that the bound ³⁵S was displaced by the addition of more cystine. Assuming that human and sheep plasma proteins react in the same way with cystine, the probable explanation of these results is that different experimental conditions were used. Eagle, Oyama, and Piez used human serum which had been saturated with labelled cystine and dialysed before treatment with non-radioactive cystine. Under these conditions the concentration of thiols would have been negligible compared with that in normal serum, thus demonstrating that there is no exchange between cystine and the mixed disulphide, PrSSCy. In the present experiments, in which the sheep plasma was not altered except for the addition of small amounts of cystine, the exchange probably occurred by reversal of equation (1), since a small concentration of cysteine is probably all that is required. The cystine in normal plasma *in vitro* thus undergoes a continuous exchange between the free and the protein-bound forms.

. Most of the published observations on thiol-disulphide exchanges have been made on systems *in vitro*, but it is probable that such exchanges occur *in vivo* as well. Jenson (1959) concluded that there are strong indications that such interchanges play a role in certain important physiological processes, including keratin production; and Eldjarn and Pihl (1957) stated: "Provided the reaction rates are relatively high, any variation in the ratio of total -SS- to total -SH effected by oxidation or reduction of any one component will necessarily affect the concentration of all other molecular species. This presumably is the situation in cells and tissues in which numerous thiols and disulphides may exist in a dynamic equilibrium". Apparently, however, no attempts have been made to demonstrate that such an equilibrium does exist *in vivo* or to study the rate of such exchanges under normal physiological conditions. The most reasonable interpretation of the results reported here is that L-[³⁵S]cystine rapidly equilibrates with the circulating free cyst(e)ine, that some of the [³⁵S]cyst(e)ine becomes bound to the plasma proteins *in vivo* (Fig. 4) as well as *in vitro* by disulphide exchange, and that this exchange occurs continuously with a half-time of about 1-2 hr in the circulating plasma (Figs. 4 and 5). Some of the ³⁵S presumably disappeared by equilibration of the injected plasma proteins with the extravascular pool but this is a relatively slow process, with a half-time of about 0.5-1 day, in other species (Sterling 1951; Cohen *et al.* 1956). The half-time of 1-2 hr in the present case is therefore mainly attributed to the thiol-disulphide exchange. The bound cystine has a much slower turnover rate than free cystine which has a half-time in plasma of only a few minutes at the most (Fig. 5). The recovery from the oxidized plasma proteins of most of the ³⁵S bound *in vivo* as cysteic acid shows that this ³⁵S was still in the form of half-cystine residues. The results in Figure 4 show that some binding occurs *in vivo*, but do not enable the concentration of bound cystine to be calculated.

Eagle, Oyama, and Piez (1960) did not discuss the possibility that some half-cystine residues are bound by the plasma proteins *in vivo* but from their data it may be calculated that the sera which they used may have contained disulphide-bound cystine *in vivo* at concentrations of at least 0.005–0.04 mM. This is based on three of their observations: (a) that freshly drawn serum or plasma contained free cyst(e)ine at

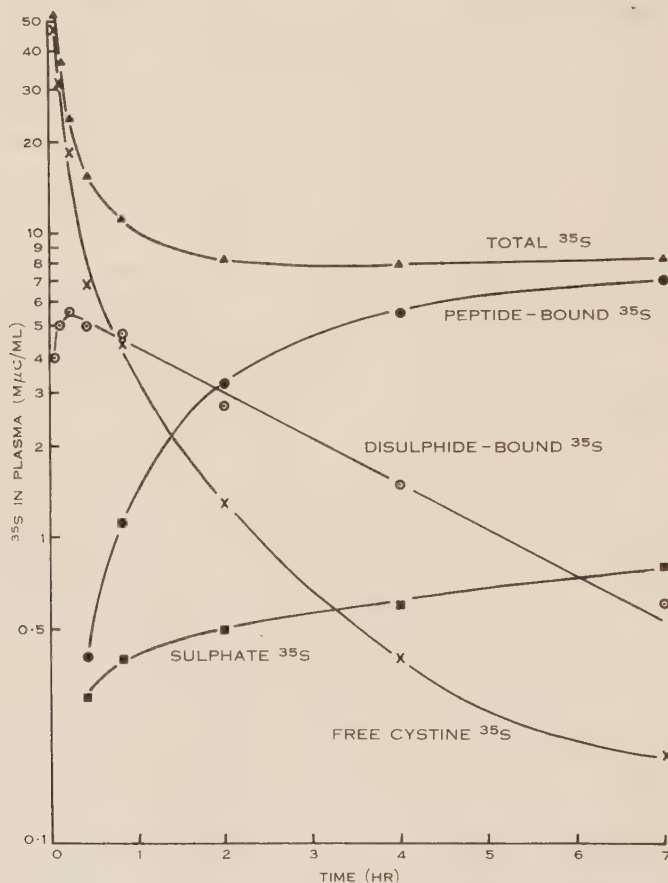


Fig. 4.—Distribution of ^{35}S in the plasma of a sheep after an intravenous dose of L- ^{35}S cystine. L- ^{35}S cystine (356 μC ; 6.7 mg) was injected into a Corriedale ewe. Blood samples were taken at intervals, cooled immediately, and centrifuged at 5°C. The plasma was analysed within 1 hr after taking each blood sample to minimize the binding of ^{35}S cystine by the proteins *in vitro*.

The results show that disulphide binding occurs *in vivo*.

a concentration of 0.04–0.045 mM (as cystine); (b) that when horse or human serum, which had been stored at -20°C for 1–8 weeks and then dialysed, was heated to 100°C , a supernatant fluid containing 0.03–0.05 mM free cystine was obtained; and (c) that a maximum of 62% of the ^{35}S was released on heating plasma containing disulphide-bound ^{35}S cystine at pH 7–9 to 100°C . From the second and third of these statements the total concentration of bound cystine in the aged serum must have been at least 0.05–0.08 mM, a concentration higher than that of the free cystine

in fresh serum by 0.005–0.04 mM. This conclusion must be regarded as being tentative only, because of the usually large experimental error of such determinations.

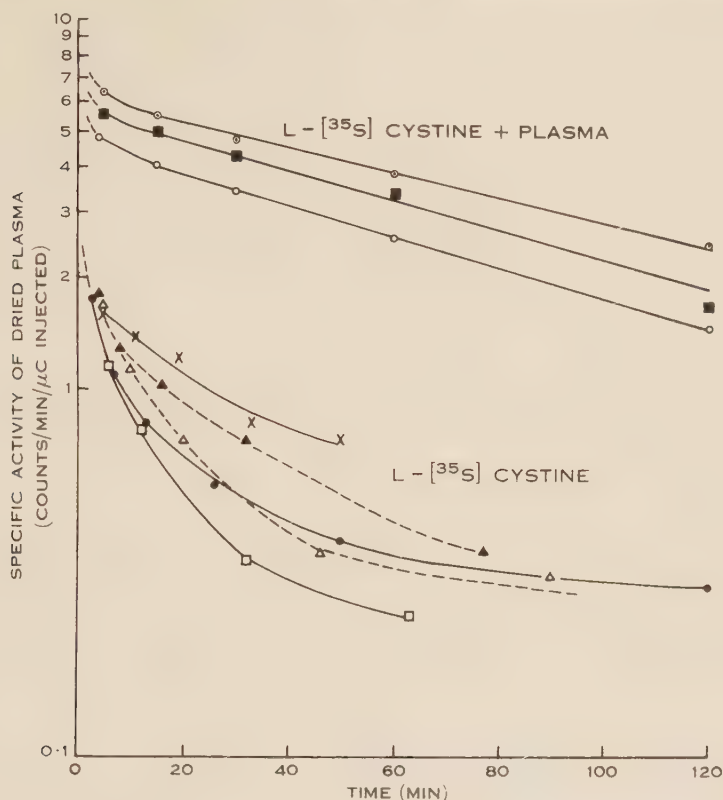


Fig. 5.—Rate of disappearance of ^{35}S from blood plasma after intravenous doses of L- ^{35}S cystine, given as either free cystine or as cystine mixed with plasma long enough to ensure disulphide binding of 60% of the radioactivity by the plasma proteins. To compare the results more easily the counting rates of infinitely thick samples (1 cm^2) of dried plasma have been divided by the number of microcuries injected. Assuming instantaneous mixing of the dose with a plasma volume of 2 l. and a dry matter content of 7% the calculated zero point on the ordinate is about 7.

Cystine + Plasma				Free Cystine			
Sheep No.	Specific Activity (μC)	Cystine (mg)		Sheep No.	Specific Activity (μC)	Cystine (mg)	
○ MA50	25.1	0.40		● A459	356	6.7	
■ MA51	20.0	0.32		□ MA50	40.3	0.64	
○ MA52	27.3	0.43		△ MA50	40.3	100	
				▲ MA52	40.2	500	
				× MA51	40.3	2000	

Since half-cystine residues are apparently bound to the plasma proteins to some extent *in vivo* and since these residues are not removed by precipitation with picric acid, it is evident that the results obtained by the techniques of Stein and

Moore (1954) for the free cyst(e)ine levels in plasma do not represent the total amount of cyst(e)ine present in a relatively free form. The results also show that small and variable amounts of half-cystine residues are probably held by the plasma proteins isolated by fractional precipitation with either neutral salts or organic solvents. The amount of bound cystine would depend on the level of cyst(e)ine in the blood at the time of sampling, on the time that elapses before the fractionation is carried out, and on the temperature of the sample during this time. This could explain why dinitrophenyl ("DNP")-cysteic acid in addition to DNP-aspartic acid has been found in hydrolysates of DNP-equine serum albumin which had been oxidized with performic acid (Titani, Yoshikawa, and Satake 1956; Turner, Kennedy, and Haurowitz 1959) whereas DNP-aspartic acid alone has been detected in DNP-bovine and DNP-human serum albumin which had been similarly treated (Thompson 1958; Biserte 1959; Turner, Kennedy, and Haurowitz 1959; Ikenaka 1960).

In view of the fairly rapid exchange between free and bound cystine in plasma and the similarity in the percentage of the doses that appeared in the wool (Table 2) it is impossible to say from these results whether either of these forms of cystine play any special role in keratinization.

About 30% of each of the intravenous doses of L-[³⁵S]cystine was incorporated into the wool grown during the subsequent 4 weeks, whether the cystine was in the free or protein-bound form and irrespective of the mass of cystine injected, up to 2 g. This is interesting because 2 g is probably massive compared to the total pool of free and disulphide-bound cystine in the plasma and appears to be comparable with the amount of cystine required *per day* for protein synthesis in the whole animal.

There seem to be no figures for the concentration of free (or bound) cystine in sheep plasma but if we assume the level to be the same as in man, about 1.2 mg free cystine per 100 ml plasma (Stein and Moore 1954), there must be no more than about 3-4 mg of free plus disulphide-bound cystine per 100 ml plasma or a total pool of about 100 mg in the circulating plasma, that is assuming that the binding can occur *in vivo* to the same extent as *in vitro*. The amount of cystine required per day was roughly estimated as follows. If we assume about 15% of the body weight of the sheep is protein, as quoted for other species by Siri (1956), and an average turnover time of 35 days ($1.44 \times$ half-time of 26 days (Downes 1961)), the amount of protein synthesized per day by the sheep used here (about 40 kg body weight) would be $(40 \times 0.15 \times 1000)/35 = 170$ g per day. Taking 1.5 as the average percentage of cystine in the proteins of the entire animal (Block and Bolling 1951), about 2.5 g of cystine per day would be required. This is probably an upper limit because about one-third of the body protein is comprised of extracellular collagen which contains no cystine and is evidently turned over very slowly indeed (Neuberger and Slack 1953). The estimate of 2.5 g per day required for protein synthesis may be compared with an estimated 3.5 g of cystine supplied daily in the ration, as cystine plus methionine (1000 g ration per day; 10% protein containing 3.5% cystine, including methionine, and assuming it is all convertible to cystine). In addition an unknown amount of these amino acids may be synthesized by micro-organisms in the rumen. The sheep studied were producing about 6 g of clean dry

wool per day. In other words, about 600 mg of cystine per day was required for wool growth.

With such estimates in mind it was not unreasonable to expect that a large proportion of an intravenous dose of 2 g of cystine would be rapidly excreted. Therefore to explain the results (Table 2), either the rate of protein synthesis increased rapidly for a short period while the excess cystine was available or else the cystine was stored and used gradually over a longer period. The latter explanation, with a pool of at least several grams of cystine and with disulphide exchange as the mechanism, seems to be the more reasonable one. This explanation is consistent with the hypothesis that there is a metabolic pool containing cystine in the skin, perhaps in the wool follicles themselves (Downes 1961).

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A MORPHOLOGICAL AND HISTOCHEMICAL STUDY OF THE BACTERIAL DEGRADATION OF WOOL FIBRES *IN VIVO*

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Summary

It has been previously shown that a microorganism, isolated from the contents of dermal cysts artificially produced in a Merino sheep, was capable of degrading wool fibres *in vitro*. This paper considers the degradation of wool fibres *in vivo* and describes structural changes and distribution of sulphhydryl groups demonstrated by histochemical technique. The fibres are arranged in order of degree of degradation which is characterized by two mechanisms; reduction of -S-S- cross-linkages and proteolysis. The degradation proceeds to disintegration of macrofibrils.

I. INTRODUCTION

In a previous communication (Molyneux 1959) the characteristics of a microorganism capable of degrading wool fibres and the method of its isolation from the contents of an experimentally produced epithelium-lined cyst were described. The degradation of the wool fibres *in vitro* proceeded stepwise; the removal of intercellular material released free cortical cells and the removal of intracellular material released intracellular fibrils. Because of the production of pink pigment in the degradation of Merino wool fibres *in vitro* and because the microorganism was isolated from an experimental cyst, a comparison was made between the degradation of wool occurring in the cysts and the degradation of wool in "pink rot", a variety of fleece rot described by Waters (1932).

The removal, 56 and 83 weeks after implantation in a Merino sheep, of two experimental cysts in which the degradation of wool fibres had occurred *in vivo*, has made possible the examination of these fibres by electron-microscopy and staining techniques.

In this paper the fibres have been arranged in an order of degradation which is influenced by their bilateral structure. The bilateral structure of wool fibres is now well established. Horio and Kondo (1953) showed that the characteristic crimp of Merino wool fibres is related to the bilateral structure of the individual fibres. They showed that when wool fibres are dyed with a basic dye (janus green) the cortex is divided into dye-accessible (DA) and non-DA segments, the latter being situated on the inside of the curvature of the coiled or crimped fibres. They also showed that the DA segment is sensitive to weak alkali as evidenced by swelling and loss of birefringence. Mercer (1953) showed that, when fine Merino wool is supercontracted by heat and then treated with trypsin, there is preferential digestion of a segment of the

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fibre. This segment was named the orthocortex and corresponds to the DA segment of Horio and Kondo. The segment resistant to enzymic digestion, the paracortex, corresponds to the non-DA segment. Also Fraser and Rogers (1953) described the division of wool fibres into S (soft) and H (hard) segments, corresponding to the orthocortex and paracortex respectively, as judged by the marked susceptibility of the S-segment to reduction with alkaline thioglycollate.

II. MATERIALS AND METHODS

Before surgical removal of the experimental cysts, a punch biopsy of the cyst walls was made. At this time a sample of intracystic wool fibres was removed and subsequently fixed in 1% trichloroacetic acid in 80% alcohol. The whole cysts were then removed and fixed in buffered 10% neutral formalin. Paraffin-embedded sections, 8–10 μ in thickness, were prepared and representative sections were stained with haematoxylin and eosin for routine examination.

The bilateral segmentation of the fibres was examined by dyeing the sections with 0.1% methylene blue in 0.03M phosphate buffer at pH 7.4 for 30 min at 100°C, as described by Fraser and Rogers (1955a).

The location of protein-bound sulphydryl groups was demonstrated by the use of dihydroxydinaphthylidysulphide (DDD), a naphthol derivative, as described by Barnett and Seligman (1952) and Barnett (1953). As controls for the DDD reaction some sections were stained with tetrazotized diorthoanisidine alone and in others blockade of sulphydryl groups was obtained by treatment with 0.1M *N*-ethyl maleimide in phosphate buffer at pH 7.4 for 6 hr at 37°C. Sections of normal Merino skin fixed in formol saline and samples of normal Merino wool, unfixed and fixed in trichloroacetic acid–alcohol, were used as additional controls in the staining methods.

Samples of intracystic fibres, previously fixed in neutral buffered formalin, were prepared for electron-microscopy. Fibres, taken from area *a* as shown in Plate 1, Figure 1, were treated with 1% osmium tetroxide in acetate–veronal buffer at pH 7.4 for 3 hr and then embedded in "Araldite" using a modification (Rogers, personal communication) of the method described by Glauert, Rogers, and Glauert (1956). To obtain additional contrast the mounted "Araldite" sections were treated for 30 sec–2 min with lead hydroxide before microscopy, as described by Watson (1958) and Peachy (1959). A sample of wool undergoing bacterial degradation *in vitro* (Molyneux 1959) was also prepared for electron-microscopy. Partially degraded wool fibres consisting of free cortical and cuticle cells at various stages of degradation were collected from a stock Merino wool–salts culture by centrifugation, stained in osmium tetroxide as above, and embedded in "Araldite".

III. RESULTS

As shown in Plate 1, Figure 1, the cysts were packed with wool fibres which when examined were at various stages of degradation. Because wool fibres continued to grow into the cyst cavity (Molyneux and Lyne 1961), the greatest breakdown of fibres, as would be expected, occurred towards the centre of the cyst, where the oldest

fibres lie. The peripheral fibres, those adjacent to the cyst epithelial lining, showed the least degradation and, on examination with histological stains, appeared to be normal. This temporal arrangement of fibres within the cyst cavity, together with alteration in structure and histochemical staining, has allowed the intracystic fibres to be placed in an "order of degree of degradation".

(a) *Staining Reactions*

(i) *Haematoxylin and Eosin*.—Evidence of degradation in the fibres was seen in preferential staining of some fibres with eosin. The differential uptake of dye indicated the existence in these fibres of two segments, the greater segment alone being stained. In the eosinophilic segment the cellular outlines of cortical cells were distinguished, while the smaller segment remaining unstained showed no evidence of its cellular structure (Plate 1, Fig. 2). Some fibres stained completely with eosin, while others were completely unstained. The debris of fibre degradation, free cortical cells, and fibrils stained variously.

(ii) *Methylene Blue*.—In order to correlate the abovementioned segmental staining with the known bilateral structure of wool fibres, sections were dyed with methylene blue. Fibres which remained unstained with eosin, and showed no structural change, exhibited preferential uptake of basic dye and were observed to consist of two segments, the larger of which stained lightly except for cortical cell outlines and nuclear remnants which stained more heavily, as described by Fraser and Rogers (1955a).

Fibres in which partial degradation had occurred also showed segmental staining with methylene blue; the degraded larger segment which in normal fibre stained with eosin now stained heavily with methylene blue; the smaller intact segment stained lightly (Plate 2, Fig. 1). Fibres in which the degradation extended through their whole thickness stained with methylene blue, although even in many of these the segmental staining was apparent in that the larger segment stained more heavily. The uptake of dye was less as the degradation of the fibres increased. From these observations the initially degraded segment of the fibres was considered to represent the orthocortex.

(iii) *Location of Sulphydryl Groups*.—Examination for the presence of sulphydryl groups showed that, whereas the majority of intracystic fibres stained heavily, only nuclear remnants and small scattered particles stained in normal control fibres. It was found that intracystic peripheral fibres stained blue (high concentration of sulphydryl) while the more central fibres stained predominantly red (lower concentration of sulphydryl). This has been interpreted as a depletion of sulphydryl-containing material from the fibres as degradation proceeds.

A temporal arrangement of fibre degradation may be conveniently illustrated by the following stages:

Stage A: The fibre consisted of two unequal segments. In the larger segment the cell outlines and nuclear remnants stained. The cuticle was intact.

- Stage B: (cf. Plate 2, Fig. 2). In the orthocortex the periphery of the cortical cells and nuclear remnants stained more heavily blue. A number of cortical cells stained completely blue while others were only partly stained; the stain appeared to penetrate the cells from the intercellular region. At this stage isolated cells, which were unstained except for the nuclear remnant, were seen in the orthocortex. In the paracortex the nuclear remnants stained more heavily than in (A). The cuticle was intact.
- Stage C: (cf. Plate 3, Fig. 1). The larger segment stained heavily blue, so that cortical cell outlines were obscured. Isolated cells may remain unstained. Irregular red-stained areas (*R*) occurred. In the smaller segment cortical cell outlines were visible and stained a faint blue. The cuticle was intact.
- Stage D: (cf. Plate 3, Fig. 2). The cortical cells of the greater segment stained uniformly red and were often separated from one another; the intercellular material and nuclear remnants were not visible. The appearance of the smaller segment varied. For example: (*a*) there may have been no structural change and only the nuclear remnant stained blue; (*b*) intercellular material, nuclear remnants, and cortical cells may have stained blue; and (*c*) intercellular material and nuclear remnants may not be visible and the cortical cells were unstained. The cuticle, when present, generally had ruptured on the side covering the orthocortex (fibre D, Plate 3, Fig. 2) or it was absent (fibre D₁, Plate 3, Fig. 2).
- Stage E: (cf. Plate 3, Fig. 1). The cortical cells of the whole fibre stained red, the intercellular material and nuclear remnants being absent. The cuticle may be intact, may have ruptured and partly lifted from the fibre, or be entirely absent.
- Stage F: (cf. Plate 3, Fig. 2). In the intracystic debris, free cortical cells, fibrils, cuticular cells, and keratin flakes from the stratum corneum could be recognized. Free cortical cells stained red or were unstained.

(*b*) *Electron-microscopy*

An electron-micrograph of a degraded fibre such as D₁ in Plate 3, Figure 2, is shown in Plate 4, Figure 1. There is an absence of cuticle and of intercellular, intermacrofibrillar, and nuclear material. The macrofibrils remain arranged so that cortical cell outlines can be distinguished.

Plate 4, Figure 2 shows an electron-micrograph of an isolated cell (stage F of degradation). Some macrofibrils are fragmented and there is an absence of intermacrofibrillar material. Higher magnification in Plate 5 reveals a microfibrillar pattern. A conspicuous feature is the fragmentation of the macrofibrils which have an ill-defined periphery. Just beneath this periphery there appears to be a band of increased electron density. Within the macrofibrils are circumscribed areas of in-

creased density which are demarcated from the surrounding macrofibrillar structure by an area of decreased density in which the fibrillar pattern is interrupted. In Plate 5, two such areas, *a* and *b*, are prominent. A portion of area *b* has apparently been lost during preparation. It is probable that these areas of increased electron density represent areas of initial degradation. Fibres degraded *in vitro* are shown in Plate 6, Figure 1. Central degradation of the macrofibrils is prominent. Plate 6, Figure 2, shows the appearance of the cuticle which had separated from an intracystic fibre. At the outer border of the cuticle a dense band can be seen in the exocuticle. Degradation has occurred involving partial loss of structure in the region of the exocuticle and endocuticle of the cell.

IV. DISCUSSION

By the application of histochemical techniques to intracystic wool fibres, a series of stages in fibre degradation has been revealed. On the basis of distribution and relative concentration of sulphydryl groups, together with a consideration of structural changes, the probable sequence of these stages of degradation has been determined.

Control (normal) fibres when stained by the DDD procedure did not react, except in so far as nuclear remnants and various rodlets stained blue. In contrast, as degradation of intracystic wool fibres proceeds the initial staining for sulphydryl groups occurs in the intercellular region of the orthocortex (stages A and B), next the entire orthocortex begins to stain heavily for -SH groups, indicating the reduction of -S-S- linkages (stage C) while the entire fibre is stained in stage E. The decrease in sulphydryl staining intensity, from blue to red, as degradation proceeds indicates a progressive loss of sulphydryl groups from the fibre. The staining of the intercellular region between the cortical cells occurs before structural change in the fibres is apparent by light-microscopy. It is possible, however, that this staining may give a false location of sulphydryl groups as reaction products from the cell contents could diffuse into intercellular spaces as a result of digestion of the intercellular membrane complex. At stages D and E marked structural changes are shown such as rupture and loss of cuticle and loss of intercellular material and nuclear remnants, which result in the release of cortical cells. Electron-micrographs show further stages of degradation in which macrofibrils are freed by the removal of intermacrofibrillar material. The macrofibrils then begin to disintegrate and gradually digest.

Geiger *et al.* (1941) demonstrated that reduction of -S-S- linkages in wool increased its susceptibility to digestion by proteolytic enzymes. Fraser and Rogers (1953) demonstrated the susceptibility of the orthocortex of Merino wool fibre to reduction with sodium thioglycollate, while Mercer, Golden, and Jeffries (1954) showed that the orthocortex is preferentially digested by pepsin following reduction with thioglycollic acid. This pattern of degradation is seen in the initial reduction of -S-S- linkages in the orthocortex of intracystic fibres. Moreover, the contrast obtained in electron-micrographs of degraded macrofibrils stained with osmium tetroxide followed by lead hydroxide (Plate 5) is similar to that shown by Merino

fibres previously reduced with 0.5M thioglycolic acid and then stained with osmium tetroxide (Rogers 1959*b*) and suggests that some reduction of the cystine of the sulphur-rich matrix between the microfibrils has occurred.

The partially degraded intracystic fibres (Plate 4, Fig. 1; Plate 6, Fig. 2) are similar to free cuticle and cortical "cells" released from wool fibres following prolonged tryptic digestion (Mercer, Farrant, and Rees 1955). The fibre components digested include the endocuticle, intercellular material, nuclear remnants, and intermacrofibrillar material. The digestion of the latter component releases individual macrofibrils. Following oxidation and extraction of wool fibres with dilute ammonia, the abovementioned fibre components remain, and have been termed "non-keratinous" (Mercer 1953, 1955; Rogers 1959*b*). Structural degradation of fibres initially occurs in the orthocortex which, in comparison with the paracortex, has a greater amount of intermacrofibrillar material (Rogers 1959*a*, 1959*b*) and is more susceptible to proteolytic attack.

Circumscribed areas of increased electron density were observed within macrofibrils from degraded intracystic fibres (*a* and *b* in Plate 5). Similar areas have been observed by Rogers (personal communication) in normal wool fibres, reduced wool fibres treated with enzymes, and particularly in porcupine quills. These areas, which could be considered as areas of faulty organization, may be initially more susceptible to digestion than the more organized parts of the macrofibril. The presence of such areas may be responsible for the central degradation of macrofibrils shown in Plate 6, Figure 1. In Plate 6, Figure 2, the dense band seen in the exocuticle corresponds to the region of the exocuticle in human hair which is particularly resistant to tryptic and keratinolytic attack (Birbeck and Mercer 1957).

Thus evidence is presented that the degradation of intracystic fibres is characterized by two mechanisms: reduction of -S-S- linkages and proteolysis. The sequence of fibre degradation, determined by structural changes and the distribution of sulphhydryl groups, is described while electron-microscopy reveals the final disintegration of the macrofibrils.

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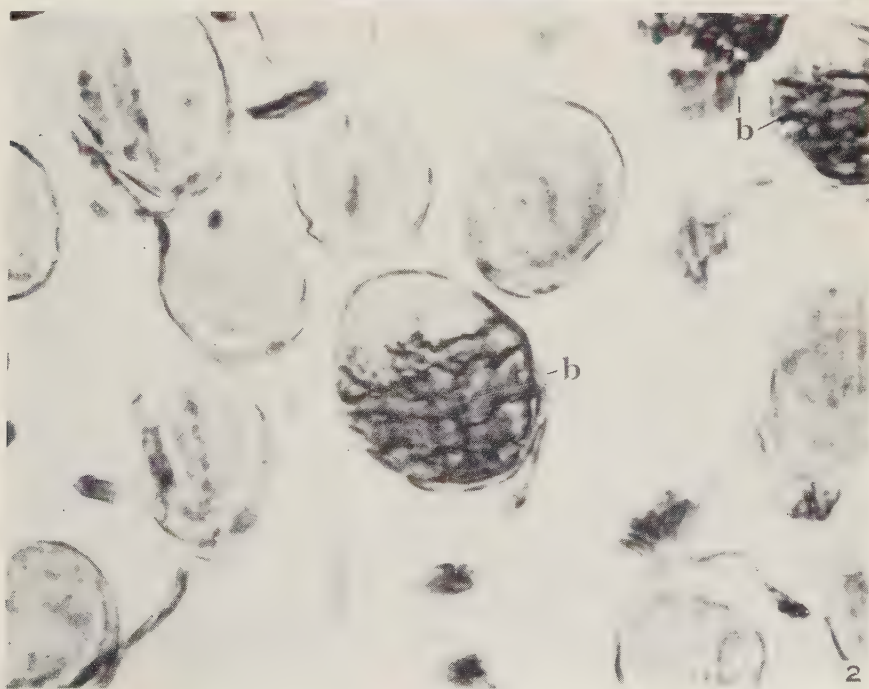
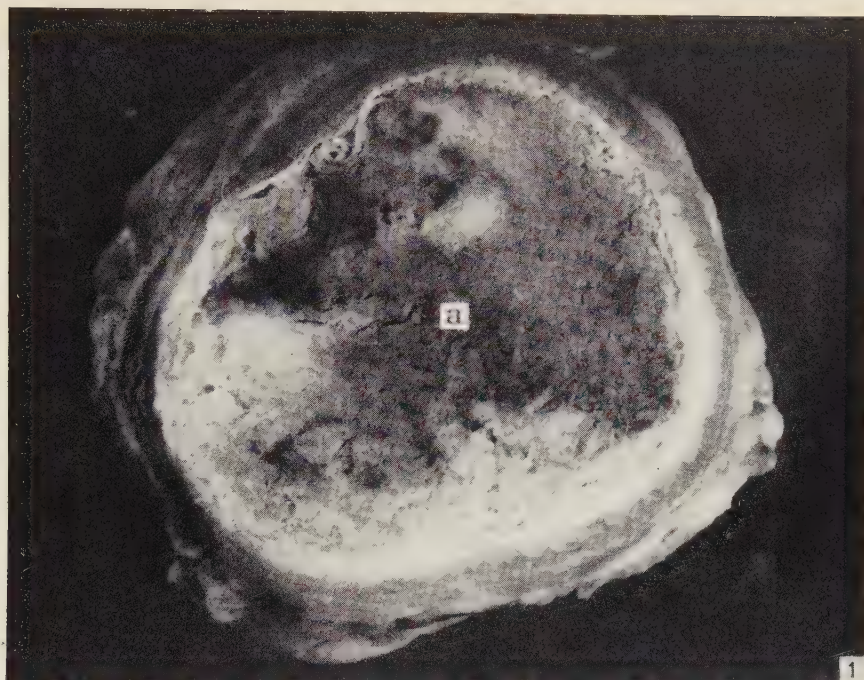
EXPLANATION OF PLATES 1-6

PLATE 1

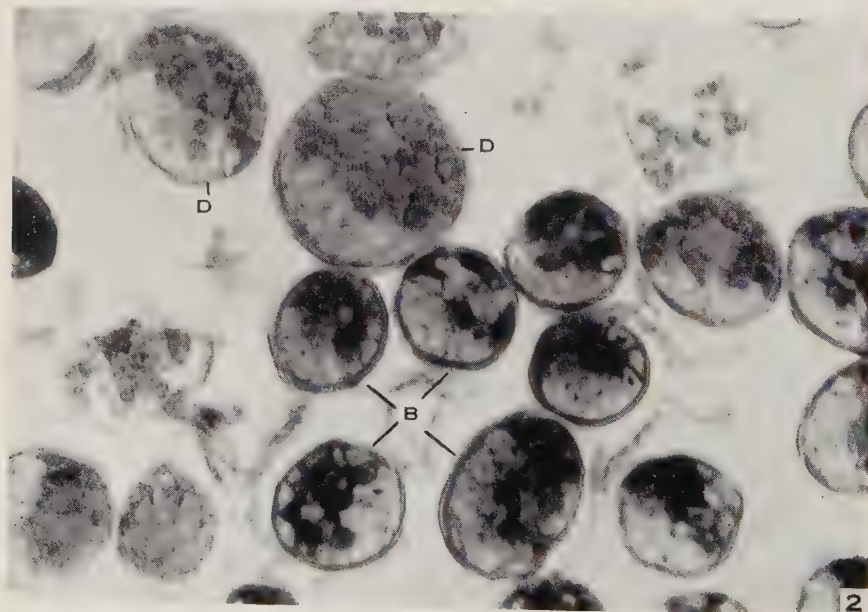
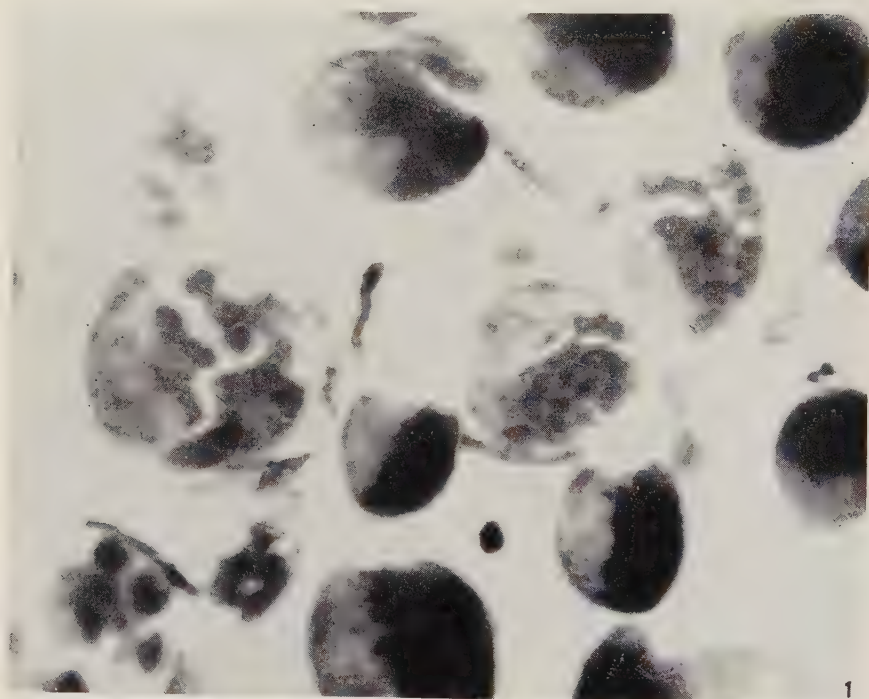
Fig. 1.—Cross section of an experimental dermal cyst removed 83 weeks after implantation. Degraded wool fibres appear dark while relatively normal fibres are situated at the periphery adjacent to the cyst wall. $\times 3.7$. *a*, Area from which fibres were taken for electron-microscopy studies.

Fig. 2.—Intracystic fibres stained with haematoxylin and eosin. *b*, Fibres showing structural change and which are stained with eosin. $\times 950$ approx.

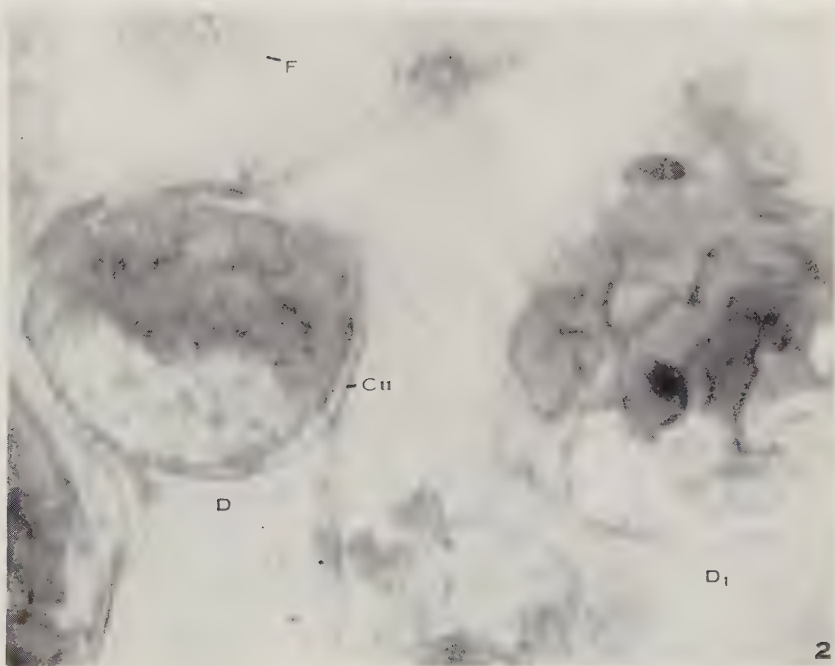
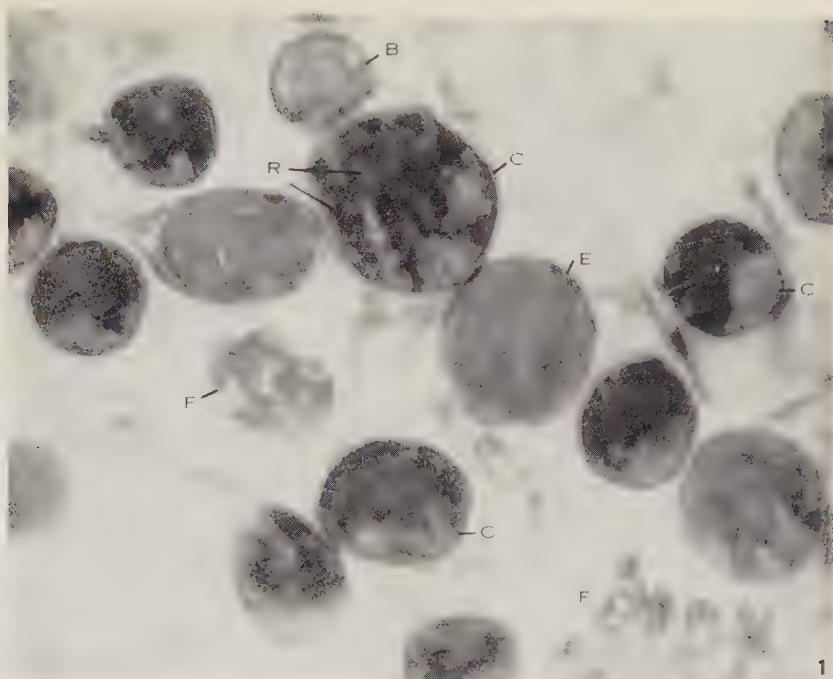
DEGRADATION OF WOOL IN VIVO



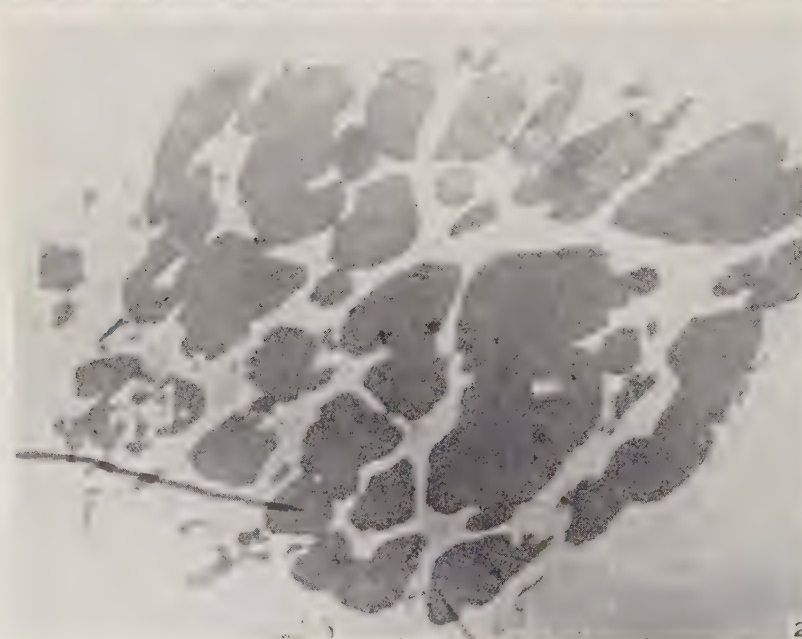
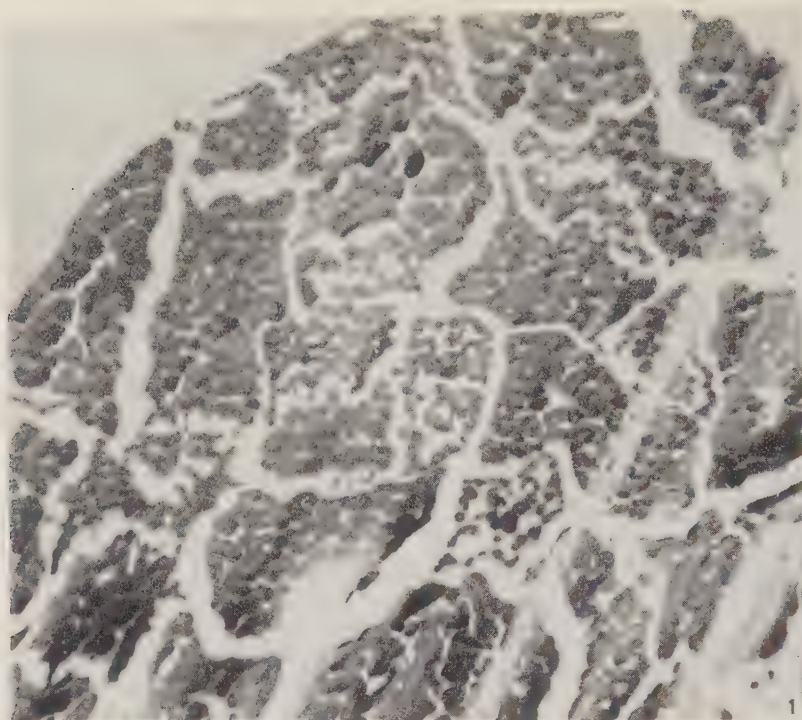
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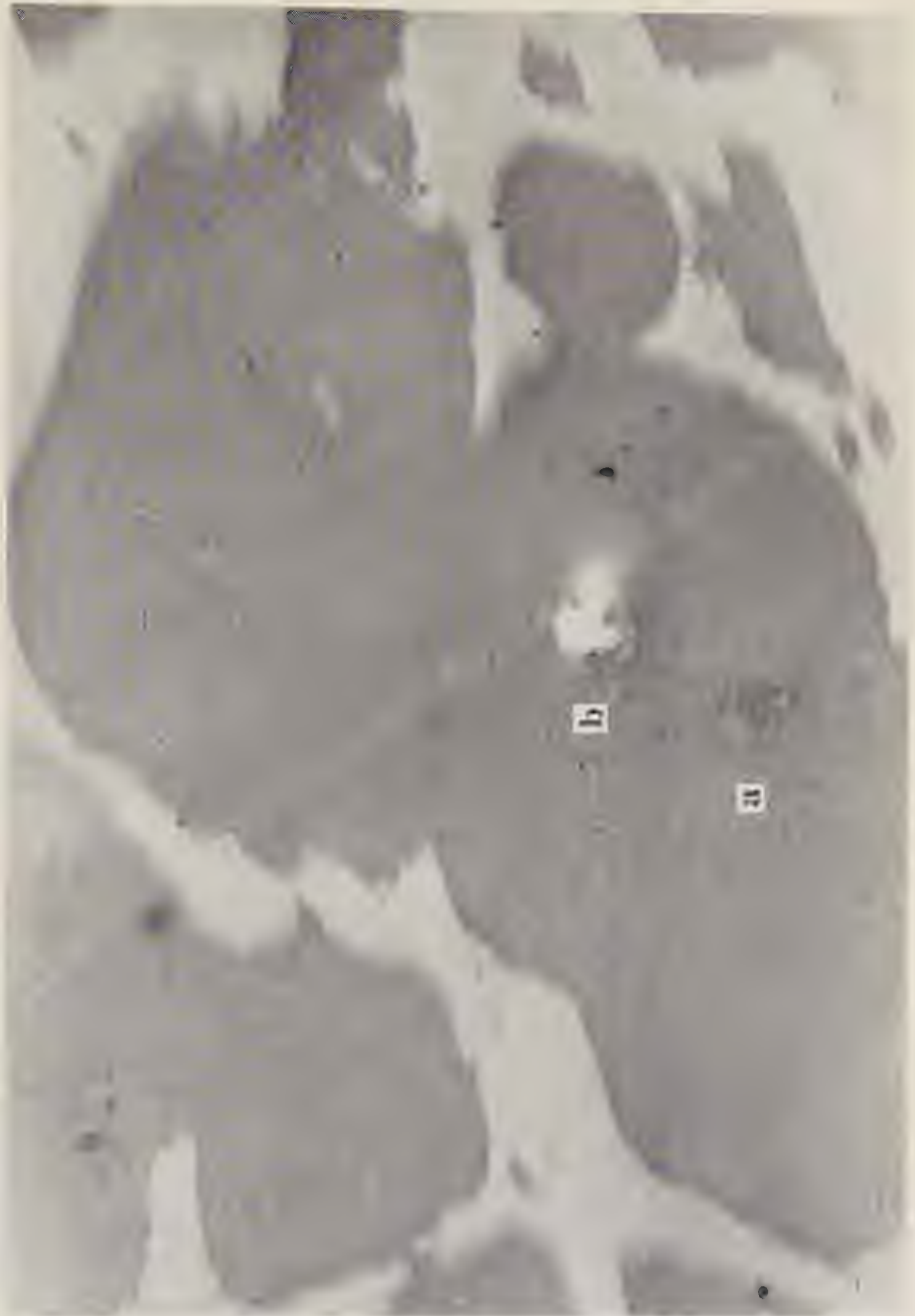


DEGRADATION OF WOOL IN VIVO



DEGRADATION OF WOOL IN VIVO





DEGRADATION OF WOOL IN VIVO

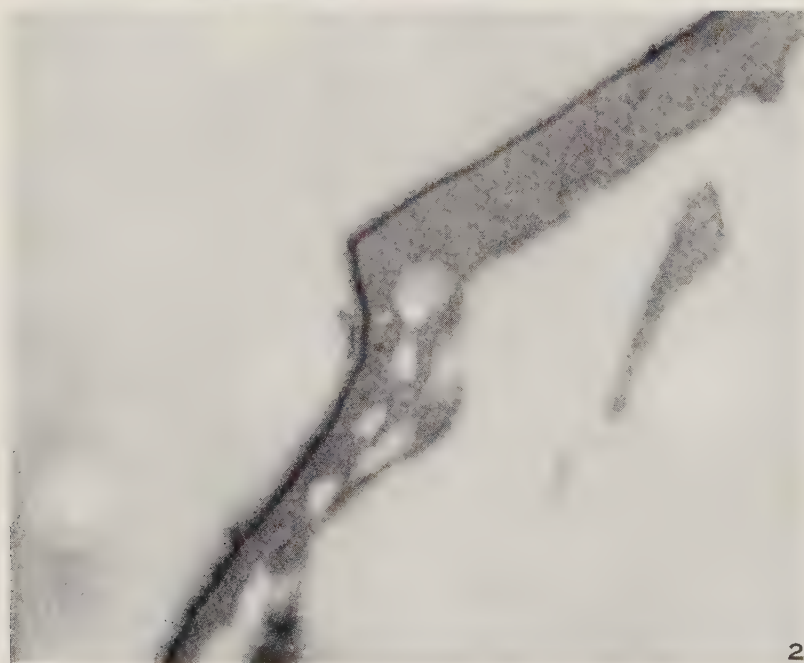
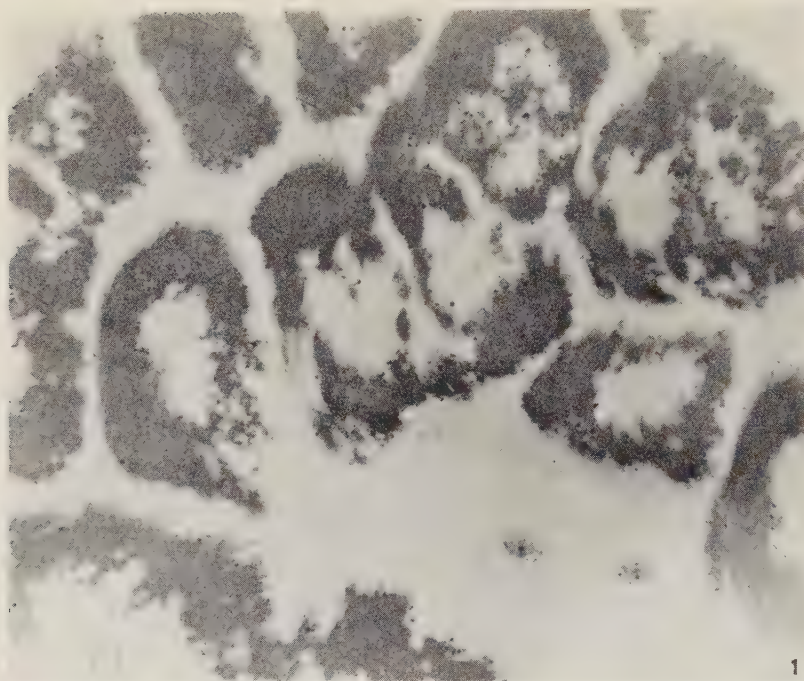


PLATE 2

- Fig. 1.—Fibres stained with methylene blue showing heavy staining of the orthocortex. Initial degradation of the orthocortex is shown by the release of cortical cells. $\times 760$ approx.
- Fig. 2.—Fibres stained for sulphhydryl groups showing stages B and D of degradation. $\times 760$ approx.

PLATE 3

- Fig. 1.—Fibres stained for sulphhydryl groups showing stages B, C, E, and F of degradation. $\times 760$ approx. *R*, irregular red-stained areas.
- Fig. 2.—Fibres stained for sulphhydryl groups showing stages D and F of fibre degradation. The cuticle has lifted from the fibre in D and is absent in D₁. The paracortex of fibre D₁ is unstained and intercellular material is absent. F shows free cortical cells. $\times 1800$ approx.

PLATE 4

- Fig. 1.—Electron-micrograph of fibre in stage D₁ of degradation. Cuticle, nuclear remnants, and intercellular and intermacrofibrillar material are absent. Cortical cell form can still be distinguished. $\times 6000$.
- Fig. 2.—Group of macrofibrils showing evidence of disintegration. $\times 21,000$.

PLATE 5

Higher-power magnification of macrofibril from Plate 4, Figure 2, showing prominent micro-fibrillar pattern. Partial fragmentation has occurred. Areas *a* and *b* suggest areas particularly susceptible to degradation within the macrofibril. $\times 84,000$.

PLATE 6

- Fig. 1.—Electron-micrograph of macrofibrils degraded *in vitro*. Fragmentation is advanced with prominent central degradation. $\times 56,000$.
- Fig. 2.—Electron-micrograph of cuticle from intracystic fibre showing a dense band in the outer region of exocuticle and partial degradation of the exo- and endocuticle. $\times 32,000$.

THE ABSORPTION OF AMMONIA THROUGH THE RUMEN OF THE SHEEP

By J. P. HOGAN*

[Manuscript received January 9, 1961]

Summary

In sheep anaesthetized with chloralose, the transport of ammonia across the rumen epithelium increases with the concentration gradient at pH 6·5. The movement of volatile fatty acids across the rumen epithelium at pH 6·5 increases the transport of ammonia. The effects both of ammonia concentration and of the movements of the fatty acids on the transport of ammonia were so reduced at pH 4·5 that they were either absent or within experimental error. The transport of ammonia was not affected within the limits of the measurements either by changes in the concentrations of sodium, potassium, chloride, carbon dioxide, or lactate in the rumen or by the net movement of water into and out of the rumen. The use of the changes in concentration of ammonia in the rumen veins to indicate transport of ammonia from the rumen is discussed.

I. INTRODUCTION

The factors that affect the transport of the ions Na^+ , K^+ , Cl^- , and the steam volatile fatty acids across the rumen epithelium of the sheep have been extensively studied in recent years. In general, it seems that the two main forces controlling the outflux of ions from the rumen to the blood are the concentration gradient and the potential difference which exists between the blood and rumen contents and which is positive for the blood (Phillipson 1955; Dobson and Phillipson 1958); these forces appear to be sufficient to account for the net fluxes of the ions studied except Na^+ . Little account has been taken of the transport of ammonia from the rumen, however, despite evidence that the movement of this substance may be both extensive and of considerable importance to the nitrogen status of the animal (McDonald 1948; Chalmers, Cuthbertson, and Synge 1954).

In the range of pH values normally found in the rumen, viz. 4·5–7 (Phillipson 1942; Briggs, Hogan, and Reid 1957), ammonia is present mainly as the NH_4^+ ion. Ammonium ions must leave the rumen against the potential gradient which is positive for the blood (Dobson and Phillipson 1958) but are assisted by the concentration gradient (McDonald 1948; Lewis, Hill, and Annison 1957). The present experiments described here, however, show that other factors also affect the transport of ammonia from the rumen. In this paper, ammonia refers to both dissociated and undissociated forms. In the discussion it is differentiated into free ammonia (NH_3) and ammonium (NH_4^+).

II. MATERIAL AND METHODS

Scottish Blackface and Merino sheep of 28–36 kg body weight, which had previously been cannulated with either ebonite or rubber cannulae in the dorsal rumen sac, were used in these experiments. The surgical techniques for the isolation of

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the rumen by ligating the oesophagus and the reticulo-omasal orifice were identical with those of Masson and Phillipson (1951). The absorption of ammonia and, in some experiments, of other ions were measured by the net changes in the solution in the rumen. The error in the recovery of solutions containing ammonia 2 min after they had been poured into the rumen in six sheep varied between $+0.7$ and -1.1% . In the following experiments, net changes less than 2% of the ammonia added were not regarded as significant.

Samples of blood were drawn by syringe from anaesthetized sheep after exposing the carotid artery and jugular and posterior (right) rumen veins. In several experiments the rate of blood flow was measured in part of the posterior rumen vein by using a simple bubble flow meter (Soskin, Priest, and Schutz 1934). Samples of blood were readily obtained through a side-arm in the apparatus.

Arterial samples were obtained in conscious sheep from exteriorized carotid loops, and venous samples through polyethylene tubing passed into the rumen veins from inside the rumen. Access to these veins was obtained through fistulae 4 in. in diameter which had been prepared in the dorsal rumen sac. These fistulae were fitted with cannulae made from polyvinyl chloride.

In several experiments the rumen was washed out and refilled with 0.9% w/v sodium chloride solution, which was left in the rumen for 60 min. During this time the concentration of ammonia in the right ruminal vein was about $3.5 \mu\text{g}$ nitrogen/ml compared with $1 \mu\text{g}$ nitrogen/ml in the peripheral circulation. Similar results were obtained with conscious sheep. In some experiments the concentration of urea nitrogen in the right ruminal vein was $1-2 \text{ mg}/100 \text{ ml}$ less than in blood from the carotid artery but in other experiments no such difference was observed. At the end of 60 min, less than 0.5 m-equiv. ammonia nitrogen could be recovered from the solution in the rumen. It was concluded that the net movement of ammonia into the rumen either as ammonia or produced from urea during an absorption experiment would not affect the results.

In two experiments, solutions of identical composition were placed in the rumen during three successive 1-hr periods. Losses of ammonia in the three periods of the first experiment were 14, 16, and 16 m-equiv., and in the second experiment 10, 9, and 9 m-equiv., respectively. These experiments indicated that the rate of transport of solutions did not vary appreciably during 3 hr. Nevertheless, all experiments were repeated several times with the order of addition of solutions randomized to avoid errors caused by changes in the preparation. Two experiments showed that the rate of transport of ammonia was the same whether ammonia was added as the sulphate, chloride, or lactate. In subsequent experiments ammonia was added as the sulphate.

Ammonia in the solutions placed in the rumen was estimated by the method of Conway (1947) or by steam distillation in a microKjeldahl apparatus. Blood ammonia was determined by the microdiffusion techniques of Conway (1947) or Seligson and Seligson (1951), the blood being measured by syringe into the diffusion vessels immediately after sampling. The corrections made by Conway for the estimation of

ammonia in human blood were used in analyses performed by the Conway method after tests had shown that the corrections were also valid for the blood of the sheep.

Total steam volatile fatty acids were estimated by the method of McAnally (1944). The pH of solutions was measured with either a Cambridge or a Pye Universal pH meter.

Sodium and potassium were determined with an "E.E.L." flame-photometer and chloride by the electrometric method of Sanderson (1952).

III. RESULTS

(a) *Effect of Concentration of Ammonia and of pH on the Rate of Transport across the Epithelium*

Many preliminary experiments indicated that, at about pH 6.5, the transport of ammonia across the rumen epithelium increased when the concentration of ammonia in the rumen rose. These results were confirmed in greater detail in two experiments (Fig. 1). Ammonium sulphate solutions were prepared in mixed phosphate

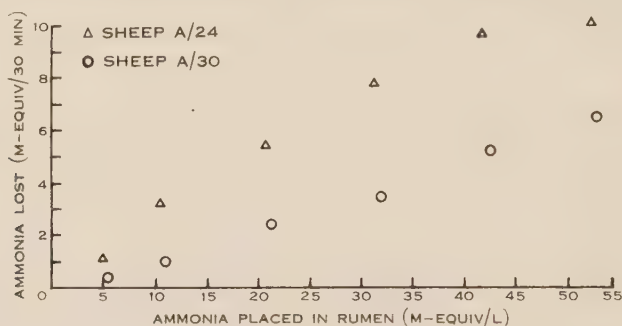


Fig. 1.—Effect of increased concentration of ammonia in the rumen at pH 6.5 on its rate of transport. The solutions placed in the rumen (2 l. of each) contained 0.002–0.026M $(\text{NH}_4)_2\text{SO}_4$, 0.018M KH_2PO_4 , 0.018M Na_2HPO_4 , 0.035M sodium acetate, and 0.004–0.077M NaCl .

and acetate buffers at pH 6.5 to give final concentrations of ammonia ranging from 3 to 53 m-equiv/l. The solutions were adjusted to 0.15M with sodium chloride, and the order in which the different solutions were placed in the rumen was randomized in each experiment. In both sheep there was an increase in the rate of transport of ammonia as the concentration of ammonia rose. The differences between the two sheep in the rates of transport of ammonia correspond to the net rates of transport of acetate, which for sheep A/24 were 27–33 m-equiv/30 min (mean 29 m-equiv.) compared with 13–15 m-equiv/30 min (mean 14 m-equiv.) for sheep A/30. There was no indication that a more rapid transport of ammonia affected the passage of the fatty acids.

Losses of ammonia again varied with concentration when solutions of ammonium sulphate ranging in concentration from 15 to 30 mM were prepared in phosphate buffers at pH 6.5 and in sucrose solutions (Table 1). These solutions were all left in the rumen for 60 min.

At pH 4.5, however, the concentration of ammonia in the rumen did not affect its rate of passage across the epithelium. Table 2 shows the results of experiments in which 15 and 30 mM solutions of ammonium sulphate were prepared in acetate and in phosphate buffers at pH 4.5. There were no consistent differences in the rate of transport of ammonia despite appreciable changes in the concentration of ammonia in the rumen.

TABLE 1
EFFECT OF AMMONIA CONCENTRATION ON THE TRANSPORT OF AMMONIA FROM PHOSPHATE BUFFERS
AT pH 6.5 AND FROM SUCROSE IN THE RUMEN

Sheep No.	Solution*	pH Range	Rumen Ammonia			
			Concentration (m-equiv/l)	In (m-equiv.)	Out (m-equiv.)	Loss (m-equiv.)
A/15	1	6.2-6.2	60	119	106	13
	2	6.3-6.2	30	60	54	6
	1	6.2-6.2	60	119	109	10
	2	6.2-6.2	30	60	59	1
A/29	1	6.5-6.3	60	119	109	10
	2	6.5-6.5	30	59	54	5
	1	6.5-6.4	60	119	109	10
	2	6.5-6.5	30	59	55	4
A/39	3	7.1-6.7	30	60	54	6
	4	6.9-6.7	60	120	107	13
	5	7.1-6.7	46	90	82	8
A/40	3	7.0-6.6	30	59	54	5
	4	6.8-6.6	60	119	110	9
	5	6.9-6.6	46	89	83	6

* Solutions (2 l. of each placed in the rumen):

- 1: 0.03M $(\text{NH}_4)_2\text{SO}_4$ + 0.038M KH_2PO_4 + 0.038M Na_2HPO_4 + 0.045M NaCl.
- 2: 0.015M $(\text{NH}_4)_2\text{SO}_4$ + 0.038M KH_2PO_4 + 0.038M Na_2HPO_4 + 0.06M NaCl.
- 3: 0.015M $(\text{NH}_4)_2\text{SO}_4$ + 0.135M sucrose.
- 4: 0.03M $(\text{NH}_4)_2\text{SO}_4$ + 0.12M sucrose.
- 5: 0.023M $(\text{NH}_4)_2\text{SO}_4$ + 0.127M sucrose.

(b) *Effect of Volatile Fatty Acids on the Transport of Ammonia*

The experiments shown in Figure 1 suggested that the movement of the volatile fatty acids from the rumen might affect the movement of ammonia. In investigating this further, use was made of the data of Masson and Phillipson (1951) who showed that the rate of transport of the volatile fatty acids across the rumen epithelium is related to their concentration. Three experiments were performed in which the

transport of ammonia from solutions of sodium acetate or butyrate at pH 6.5 were observed. The concentration of ammonia in all solutions was 30 m-equiv/l, while the concentration of volatile fatty acids varied between 0 and 100 m-equiv/l. Figure 2 shows the extent of the transport of the fatty acids and Figure 3 the relationship between the transport of fatty acids and ammonia. It may be noted that far more

TABLE 2
EFFECT OF CONCENTRATION OF AMMONIA AT pH 4.5 ON ITS RATE OF TRANSPORT FROM THE RUMEN

Sheep No.	Solution*	pH Range	Rumen Ammonia			
			Concentration (m-equiv/l)	In (m-equiv.)	Out (m-equiv.)	Loss (m-equiv.)
A/2	1	4.5-4.9	30	58	57	1
	2	4.3-5.2	60	116	110	6
	2	4.5-5.0	60	116	112	4
	1	4.6-5.0	30	59	54	5
A/3	1	4.5-4.8	30	61	54	7
	2	4.5-5.0	60	116	112	4
	2	4.5-4.9	60	121	115	6
	1	4.5-5.0	30	59	54	5
A/34	3	4.5-5.0	60	120	119	1
	4	4.5-5.0	30	60	59	1
	3	4.5-4.9	60	120	118	2
	4	4.6-4.9	30	60	58	2

* Solutions (2 l. of each placed in the rumen):

- 1: 0.015M $(\text{NH}_4)_2\text{SO}_4 + 0.03\text{M CH}_3\text{COONa} + 0.045\text{M CH}_3\text{COOH} + 0.06\text{M NaCl}$.
- 2: 0.03M $(\text{NH}_4)_2\text{SO}_4 + 0.03\text{M CH}_3\text{COONa} + 0.045\text{M CH}_3\text{COOH} + 0.045\text{M NaCl}$.
- 3: 0.03M $(\text{NH}_4)_2\text{SO}_4 + 0.056\text{M KH}_2\text{PO}_4 + 0.056\text{M NaH}_2\text{PO}_4 + 0.008\text{M NaCl}$.
- 4: 0.015M $(\text{NH}_4)_2\text{SO}_4 + 0.056\text{M KH}_2\text{PO}_4 + 0.056\text{M NaH}_2\text{PO}_4 + 0.023\text{M NaCl}$.

fatty acid than ammonia left the rumen. When only small quantities of fatty acids were present, increases in the transport of the fatty acids did not affect the movement of ammonia (Table 3).

At pH 4.5, the rate of transport of volatile fatty acids was greatly increased as the concentration rose. However, the rate of transport of ammonia did not seem to be related to the movement of the fatty acid (Fig. 4).

(c) *Effect of other Molecules*

The rate of transport of ammonia at a concentration of 30 m-equiv/l at pH 6.5 was not affected by changes in the concentration of lactate (0-80 m-equiv/l), potassium (0-135 m-equiv/l), sodium (0-135 m-equiv/l), or chloride (0-150 m-equiv/l).

When carbon dioxide was bubbled through solutions in the rumen, there was an increase in the net rate of transport of chloride from the rumen from 8 to 15 m-equiv/hr, but no change in the rate of transport of ammonia. The net movements of water into and out of the rumen were varied by altering the tonicity of the solution in the rumen. Water movements which varied between a gain of 40 ml and a loss of 170 ml showed no appreciable effect on the movement of ammonia.

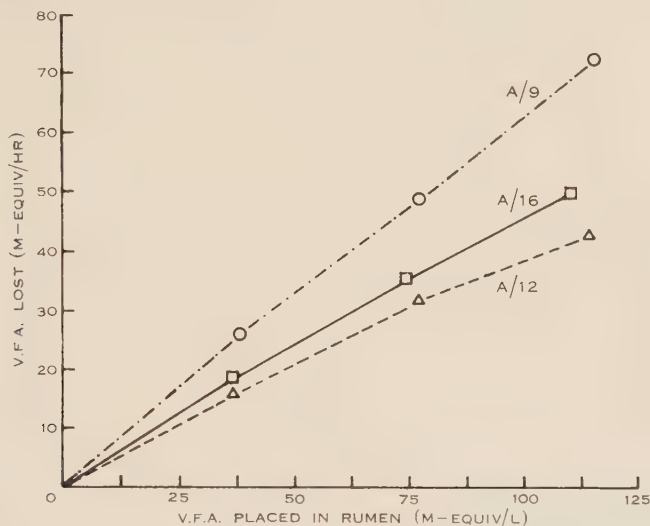


Fig. 2.—Effect of concentration of volatile fatty acids in the rumen on their rate of transport at pH 6.5. The solutions placed in the rumen (2 l. of each) contained for sheep A/9 and A/16, 0.015M $(\text{NH}_4)_2\text{SO}_4$; 0.09M KH_2PO_4 ; 0.09M Na_2HPO_4 , 0.0–0.110M sodium acetate and 0.007–0.117M NaCl. For sheep A/12 sodium butyrate replaced sodium acetate.

(d) *Rate of Transport of Ammonia in the Blood Draining the Rumen*

In a number of experiments an estimate of the quantity of ammonia carried in part of the posterior rumen vein was made while net losses of ammonia from the rumen were being measured. In Table 4 the results of two experiments are shown, in which 15 and 30 mM solutions of ammonium sulphate in Krebs–Ringer–bicarbonate buffers containing acetate were added to the rumen. The higher concentration of ammonia in the rumen was accompanied by increased concentrations in the rumen vein; the rate of blood flow was reduced but this would not be sufficient to account for the great increase in the ammonia concentration. In both experiments the concentration of ammonia in the rumen vein indicated qualitatively the rate of transport of ammonia across the rumen epithelium. The third section of Table 4 presents results of analyses performed on blood from the posterior rumen vein of a conscious sheep. The concentration of ammonia nitrogen increased from 5 $\mu\text{g/ml}$ blood when phosphate buffers alone were in the rumen to 18 $\mu\text{g/ml}$ when the concentration of ammonia in the rumen was brought to 20 m-equiv/l; when the concentration of ammonia in the rumen was further increased to 40 m-equiv/l, the

concentration of blood ammonia nitrogen rose to $29 \mu\text{g/ml}$. These results suggest that the observations made on the anaesthetized sheep above are qualitatively similar to those which occur in a standing conscious sheep.

When the rumen pH was altered with acetate and phosphate buffers, however, there did not seem to be such a clear relationship between net losses of ammonia from the rumen and the concentration of ammonia in the rumen vein. Table 5 shows the results of two experiments. With sheep S/32 the addition of the more acid solution to the rumen was accompanied by an appreciable fall in the concentration of

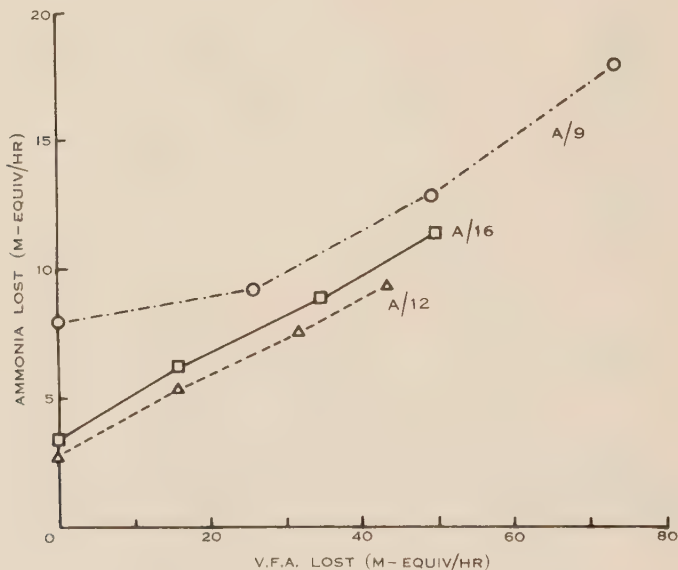


Fig. 3.—Effect of volatile fatty acid transport on ammonia transport from the rumen at pH 6.5. The solutions used were as described for Figure 2.

ammonia in the blood; however, the rate of blood flow was so greatly increased that the movement of ammonia in this part of the rumen vein was much the same in both absorption periods. This result did not reflect the net losses from the rumen. In sheep S/34 the replacement of a solution in the rumen at pH 4.5 with another at pH 6.5 produced a slight increase in the rate of flow of blood in the rumen vein. This was not expected in view of previous results (Dobson and Phillipson 1956). It may be seen, however, that the concentration of ammonia in the vein was simultaneously increased and that the ammonia carried in this part of the vein gave some indication of the net losses of ammonia from the rumen. It appears from these two experiments that the use of the concentration of ammonia in the rumen vein to indicate qualitatively the transport of ammonia from the rumen could be misleading under conditions where the rate of blood flow in the vein might alter appreciably.

IV. DISCUSSION

Most investigations of the transport of ions across the rumen epithelium have referred to the net flux—the balance between the outflux of the ions from the rumen to the blood and the influx from the blood to the rumen. This is true for sodium, potassium, and chloride, the concentration of which in arterial blood may be similar to that in rumen contents; however, it should not apply to ammonia, the concentration of which in arterial blood is not normally more than 0·1 mM (McDonald 1948;

TABLE 3
EFFECT OF SMALL QUANTITIES OF VOLATILE FATTY ACIDS (V.F.A.) ON THE TRANSPORT OF AMMONIA
Sheep No. A/21

Solution*	pH	Ammonia Nitrogen (m-equiv.)			V.F.A. (m-equiv.)		
		In	Out	Loss	In	Out	Loss
1	6·3–6·0	181	169	12	0	0	0
2	6·4–6·5	181	169	12	3·4	2·6	0·8
3	6·5–6·4	180	169	11	10·4	8·5	1·9
4	6·5–6·4	180	171	9	7·0	5·4	1·6

* Solutions (2 l. added to the rumen):

1: 0·045M $(\text{NH}_4)_2\text{SO}_4$ + 0·038M Na_2HPO_4 + 0·038M KH_2PO_4 + 0·029M NaCl .

2: 0·045M $(\text{NH}_4)_2\text{SO}_4$ + 0·038M Na_2HPO_4 + 0·038M KH_2PO_4 + 0·027M NaCl + 0·002M CH_3COONa .

3: 0·045M $(\text{NH}_4)_2\text{SO}_4$ + 0·038M Na_2HPO_4 + 0·038M KH_2PO_4 + 0·023M NaCl + 0·006M CH_3COONa .

4: 0·045M $(\text{NH}_4)_2\text{SO}_4$ + 0·038M Na_2HPO_4 + 0·038M KH_2PO_4 + 0·025M NaCl + 0·004M CH_3COONa .

Lewis, Hill, and Annison 1957). Ammonia from the blood would have to enter the rumen against an appreciable concentration gradient, for the concentration of ammonia in the rumen usually lies between 7 and 60 m-equiv/l (McDonald 1948; Annison *et al.* 1954).

Some ammonia may be formed in the rumen during an experiment, however, for it appears from arteriovenous differences that urea is at times removed from the blood as it passes through the rumen. That some of this urea is converted to ammonia is suggested by the fivefold concentration of ammonia in the right ruminal vein above the arterial level in both conscious and anaesthetized sheep whose rumens have been emptied, washed out, and filled with 0·9% sodium chloride solution. Nevertheless, as the concentration of ammonia in the rumens of these sheep, even after an hour, did not exceed 0·2 m-equiv/l, it is assumed that rumen ammonia derived either directly from ammonia or indirectly from urea in the blood would not affect the interpretation of the present experiments. The losses of ammonia from the rumen recorded in these experiments, while in reality a net flux, may therefore be regarded

as an outflux from the rumen to the blood. Since these experiments were completed, Houpt (1959) has described experiments in which ammonia accumulated at the rate of 2 m-mole/hr in the washed-out, isolated rumen. These figures are about four times as high as those found here. In the absence of information on the diets of Houpt's sheep or their feeding routine before the rumen was emptied, it is not possible to discuss the differences in the two sets of results. The important point, however, is that Houpt

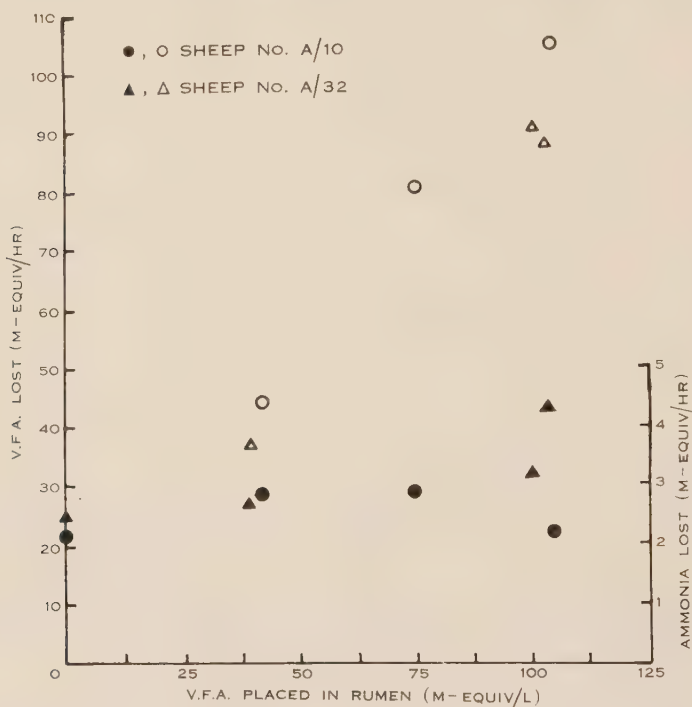


Fig. 4.—Effect of concentration of volatile fatty acids at pH 4.5 on the rate of transport of fatty acids (○, △) and of ammonia (●, ▲) from the rumen. The solutions placed in the rumen (2 l. of each) contained 0.015M $(\text{NH}_4)_2\text{SO}_4$, 0.019M KH_2PO_4 , 0.019M NaH_2PO_4 , 0.054M acetic acid, 0.044M sodium acetate, and 0.098M NaCl.

observed that ammonia accumulated at a relatively steady rate during several hours. This confirms our idea that ammonia would enter the rumen during each of the four periods of an absorption experiment to about the same extent, and thus the "endogenous" production of ammonia during an absorption experiment would not affect the comparisons of the transport of ammonia during the individual absorption periods.

The rate of transport of ammonia across the rumen epithelium was increased by raising the concentration of ammonia in the rumen from 30 to 60 m-equiv/l at pH 6.5. This occurred when the other constituents in the rumen were either acetate which is readily transported across the epithelium, phosphate which is usually

transported only slowly (Scarisbrick and Ewer 1951), or sucrose. However, at pH 4.5, the rate of transport of ammonia from the rumen was not altered appreciably with changes in concentration. It thus appears that the change in pH from 6.5 to 4.5 affected the mechanism of transport of ammonia from the rumen. This could have occurred either by altering the rumen epithelium in some way or by alterations in the ionic composition of the solutions.

TABLE 4

RATE OF FLOW OF BLOOD AND THE CONCENTRATION OF AMMONIA IN PART OF THE RIGHT RUMINAL VEIN OF THE SHEEP

Sheep No.	Solution*	Rumen Ammonia (m-equiv/l)	Rumen pH	Rate of Blood Flow (ml/min)	Concn. of Ammonia Nitrogen in Rumen Vein ($\mu\text{g/ml}$)	Total Ammonia Nitrogen in Blood (mg/min)	Net Loss of Ammonia Nitrogen from Rumen (m-equiv/hr)
S/27	1	30	6.2-6.9	19	29	0.55	10
	2	60	6.6-7.0	14	53	0.74	24
	1	30	6.6-6.9	19	29	0.55	7
S/30	1	30	6.3-7.0	20	14	0.28	9
	2	60	6.3-6.9	12	35	0.42	15
	1	30	6.5-6.9	16	24	0.38	2
G/77†	3	0	6.5	—	5		
	4	20	6.5	—	18		
	5	40	6.5	—	29		

* Solutions (4 l. for S/27 and S/30, 2 l. for G/77):

1: 0.015M $(\text{NH}_4)_2\text{SO}_4$ + 0.021M CH_3COONa + 0.114M Krebs-Ringer-bicarbonate.

2: 0.03M $(\text{NH}_4)_2\text{SO}_4$ + 0.021M CH_3COONa + 0.099M Krebs-Ringer-bicarbonate.

3: 0.075M KH_2PO_4 + 0.075M Na_2HPO_4 .

4: Solution 3 + 0.01M $(\text{NH}_4)_2\text{SO}_4$.

5: Solution 4 + 0.01M $(\text{NH}_4)_2\text{SO}_4$.

† Conscious sheep.

At the pH of the present experiments, almost all the ammonia would be present as NH_4^+ . However, some free ammonia is always present in equilibrium with NH_4^+ according to the equation $\text{NH}_4^+ \rightleftharpoons \text{H}^+ + \text{NH}_3$. The proportions of NH_3 and NH_4^+ may be calculated from the Henderson-Hasselbach equation which Bromberg, Robin, and Forkner (1960) have shown approximates to

$$\text{p}K_a = \text{pH} + \log (\text{total ammonium}/\text{NH}_3).$$

These authors estimated the $\text{p}K_a$ of this system, in anaesthetized dogs, to be 9.15.

From their figures it may be calculated that the ratio total ammonium: NH_3 in plasma at pH 7.4 is 56:1 and in rumen contents at pH 6.5 and 4.5, 450:1 and 45000:1 respectively. Plasma, which has a total ammonium concentration of 100 μg nitrogen/100 ml or 71 $\mu\text{-equiv/l}$ would thus have 1.25 $\mu\text{-equiv/l}$ NH_3 . Rumen fluids containing 30 m-equiv/l ammonium at pH 6.5 and 4.5 would have respectively 67 $\mu\text{-equiv/l}$ and 0.67 $\mu\text{-equiv/l}$ NH_3 . Thus while there is a much greater concentration of NH_3 in the rumen at pH 6.5 than in the blood, at a rumen pH of 4.5 the reverse obtains.

TABLE 5

EFFECT OF LOWERING THE pH OF RUMEN CONTENTS ON THE RATE OF FLOW OF BLOOD AND THE CONCENTRATION OF AMMONIA IN THE RIGHT RUMEN VEIN

Sheep No.	Solution*	Rumen pH	Rate of Blood Flow (ml/min)	Ammonia Nitrogen in Venous Blood Leaving Rumen ($\mu\text{g/ml}$)	Total Ammonia Nitrogen in Blood (mg/min)	Net Loss of Ammonia Nitrogen from Rumen (m-equiv/hr)
S/32	1	6.4-6.6	26	31	0.81	14
	2	4.4-4.8	55	11	0.60	6
	1	6.4-6.5	22	29	0.64	11
S/34	2	4.9-5.8	49	14	0.69	1
	1	6.5-6.8	56	28	1.57	17
	2	4.4-5.6	32	16	0.51	4

* Solutions (4 l. of each):

1: 0.015M $(\text{NH}_4)_2\text{SO}_4 + 0.075\text{M}$ $\text{CH}_3\text{COONa} + 0.008\text{M}$ $\text{HCl} + 0.021\text{M}$ $\text{KH}_2\text{PO}_4 + 0.031\text{M}$ Na_2HPO_4 .

2: 0.015M $(\text{NH}_4)_2\text{SO}_4 + 0.038\text{M}$ $\text{CH}_3\text{COONa} + 0.038\text{M}$ $\text{CH}_3\text{COOH} + 0.03\text{M}$ $\text{HCl} + 0.01\text{M}$ $\text{KH}_2\text{PO}_4 + 0.019\text{M}$ Na_2HPO_4 .

These calculations suggest that one mechanism for the transport of ammonia across the rumen epithelium is by diffusion as NH_3 . They also indicate why ammonia is transported from the rumen in accordance with its concentration gradient at pH 6.5 but not at pH 4.5.

The effect of the fatty acids at pH 6.5 on the transport of ammonia appeared only when large quantities of acid were being absorbed. The absence of any "mole for mole" relationship indicated that there was only an indirect effect on ammonia transport. At pH 6.5, most of the fatty acid would be present as the anion and it has been suggested that it is absorbed as such (Danielli *et al.* 1945). It is known (Masson and Phillipson 1951) that when a volatile fatty acid anion leaves the rumen it is replaced by an influx of bicarbonate. The quantity entering is about half the fatty acid disappearing. The remainder of the fatty acid presumably passes from the rumen accompanied by a corresponding number of positively charged ions such as the ammonium ion.

The rate of transport of ammonia from the rumen at pH 4.5 was not appreciably affected by the movement of the volatile fatty acids. At this pH, most of the fatty acid crosses the rumen as free fatty acid (Danielli *et al.* 1945) which would require neither the movement of cations nor exchange with anions.

In the absence of volatile fatty acids the transport of ammonia from solutions containing 30 m-equiv/l ammonia in the rumen was usually small. However, ammonia was lost from the rumen in every experiment. That this was a real transport across the epithelium and not merely adsorption onto it is shown by the 10- to 40-fold increase in the concentration of ammonia in the rumen veins as soon as solutions containing ammonia were added to the rumen. The rise in blood ammonia was so extensive that it could not be accounted for merely by a decrease in the rate of flow of blood through the vein, and indeed, a reduction in the rate of blood flow was observed only when acetate was also present in the rumen at about pH 6.5.

Attempts were made to use the change in concentration of ammonia in the rumen vein as a semiquantitative indication of changes in the rate of transport of ammonia across the rumen epithelium. This method, if successful, would have been a more sensitive indication of transport from the rumen than net losses measured with large volumes of fluid. However, the observation that the addition of ammonia to the rumen was at times accompanied by a drop in the rate of flow of blood in the rumen vein, suggested that measurements of the concentration of ammonia without an accurate estimate of the rate of flow of the blood could be meaningless.

The movement of ammonia across the rumen epithelium was observed to be independent within experimental error of variations in the concentration in the rumen of sodium, potassium, lactate, chloride, and carbon dioxide, and of the net movement of water into and out of the rumen.

While it appears from the present experiments that, of all the substances crossing the rumen epithelium, the volatile fatty acids alone affect the transport of ammonia, it must be remembered that these results record virtually an outflux of volatile fatty acids and ammonia but a net flux of other ions. The present conclusions on the factors affecting the transport of ammonia must therefore be regarded as tentative until the estimation of the true fluxes of other ions may be made.

V. ACKNOWLEDGMENTS

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STUDIES ON OXIDIZED WOOL

IV. FRACTIONATION OF PROTEINS EXTRACTED FROM WOOL ON DEAE-CELLULOSE USING BUFFERS CONTAINING 8M UREA

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Summary

α - and γ -keratases, which are soluble proteins extracted from oxidized wool, give widely spread zones in their elution curves when examined by gradient elution chromatography on columns of diethylaminoethyl (DEAE)-cellulose in buffers containing 8M urea. Stepwise elution of the proteins indicates that they could consist of many components. An arbitrary chromatographic fractionation of α -keratase into three components followed by amino acid analysis of the fractions revealed distinct differences in amino acid composition among the components. No evidence was obtained for the presence of a major homogeneous protein component in oxidized wool. It is postulated that the original wool itself may consist of families of closely related proteins.

I. INTRODUCTION

In the early stages of fractionation of a mixture of proteins, chromatography by stepwise or gradient elution can often be applied successfully. In the case of aggregated or unfolded protein molecules (and often in the case of native protein molecules) it is frequently found that binding to the adsorbent is not truly reversible. In such cases it is probable that differences in states of aggregation or even minor variations in the shape of the protein may result in the elution of the protein in several peaks (cf. Shapira and Parker 1960) which could erroneously be considered a fractionation into proteins of different chemical constitution. One way of limiting such complexity in elution behaviour is to carry out the chromatography with buffers containing disaggregating agents.

In our experiments we have used buffers containing 8M urea in an attempt to minimize complications which may arise in the effluent patterns due to aggregation of the protein molecules. 8M urea has made it possible not only to chromatograph insulin on "Amberlite IRC-50" (Cole 1960) and diethylaminoethyl (DEAE)-cellulose (Thompson and O'Donnell 1960) but has given resolution not possible in the absence of urea. With many proteins (Cole and Mendiola 1960) the presence of 8M urea enables chromatography to be carried out at pH values where the protein normally may be insoluble.

α -keratase and γ -keratase are acidic proteins isolated from oxidized wool (Alexander and Hudson 1954; O'Donnell and Thompson 1959). α -keratase is aggregated in aqueous solution (O'Donnell and Woods 1956*a*, 1956*b*) and it was desirable to study chromatographic separation of this material and γ -keratase under the

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best disaggregating conditions possible. Buffers containing 8M urea are suitable for disaggregating wool proteins (Woods 1959).

This paper reports the fractionation of α - and γ -keratases on columns of DEAE-cellulose using stepwise elution with buffers containing 8M urea. Amino acid analysis has been used to show that the fractionation of α -keratase involves molecules of different chemical composition.

II. EXPERIMENTAL

(a) *Preparation of Proteins*

The α - and γ -keratases were extracted at pH 8 from wool (obtained from a single fleece, MW118, 64's quality) oxidized with performic acid. They were freeze-dried from aqueous salt-free solution, or else stored in frozen aqueous solution and freshly freeze-dried before chromatography. The α - and γ -keratases extracted under these conditions represent only 33 and 16%, respectively, of the wool but it is believed that these are not substantially different from the larger amounts extractable at higher pH values (O'Donnell and Thompson 1959).

Insulin and bovine plasma albumin were oxidized with performic acid reagent at 0°C and isolated by freeze-drying twice from aqueous solutions (Hirs 1956).

(b) *Operation of DEAE-cellulose Columns*

The columns of DEAE-cellulose (0.9 by *c.* 15 cm) were prepared and packed in the 8M urea buffer as described previously (Thompson and O'Donnell 1960). The DEAE-cellulose was used in predominantly the chloride form, being regenerated with 1M potassium chloride in 8M urea buffer at pH 7.4. It was periodically regenerated with 1N sodium hydroxide solution (Sober *et al.* 1956). The protein was eluted either by stepwise or gradient elution and, for the latter, a mixer with a 120-ml mixing chamber of the type described by Moore and Stein (1954) was used, the gradient being calculated according to Bock and Ling (1954). The volume from the outlet of the mixing chamber to the top of the column was 17 ml. Polyethylene tubing was used throughout since the 8M urea buffer dissolved large amounts of ultraviolet-absorbing material from polyvinylchloride tubing. All experiments were carried out at 25°C with water-jacketed columns. The recovery of proteins from the columns was greater than 90% but with the widely spread curves of the extracted wool proteins the value cannot be obtained precisely.

(c) *Buffer and Protein Solutions*

The columns were in all cases washed with the initial buffer consisting of 8M urea (B.D.H. "Analar"), 0.01M Tris (tris(hydroxymethyl)aminomethane), and 0.001M "Versene" adjusted to pH 7.4 until the pH values of the influent and effluent were identical. After regeneration with 1M potassium chloride in 8M urea solution it was necessary to pass at least 140 ml of starting buffer through the column. The buffer was filtered through a separate column of DEAE-cellulose before use. It was used within 2-3 days and stored at 2°C when not in use. The gradient was usually formed with 1.1M potassium chloride in the upper chamber of the mixer.

The freeze-dried proteins were dissolved in the buffer and in some cases dialysed overnight against the initial buffer although this made no difference to the elution pattern of the protein. Usually 10 mg of protein in 0.5 ml buffer were applied to the column.

(d) *Analysis of Effluent Fractions*

Approximately 1.1-ml fractions of effluent were collected by means of a drop counter. These were diluted with 3 ml of water before measuring their optical density against a water blank in 1-cm cells at 276 m μ . No correction was made for scattering of light.

(e) *Cysteic Acid Analyses*

These were carried out as described in a previous paper (Thompson and O'Donnell 1959) except 1 ml (instead of 3 ml) of the solutions containing cysteic acid was loaded on the column.

(f) *Analysis for Basic Amino Acids*

These were carried out on a 15-cm long column of "Dowex 50-X8" (200-400 mesh) according to the method of Moore and Stein (see Moore, Spackman, and Stein 1958). The protein hydrolysate was prepared by hydrolysis of approximately 40 mg of protein in 10 ml redistilled hydrochloric acid (6N) under reflux for 22 hr in an oil-bath at 138°C. This was then made up to 50 ml with water.

Aliquots (5 ml) were freeze-dried and the residue dissolved in 3 ml of citrate buffer (pH 2.2) and 2 ml of this was loaded on the column. 2-ml fractions were collected, the column being developed with pH 5.28 buffer as described by Moore, Spackman, and Stein (1958). The effluent fractions were analysed using a ninhydrin method. To each 2-ml fraction was added 0.2 ml of a 10% solution of recrystallized ninhydrin (Moore and Stein 1948) in purified methyl "Cellosolve" followed by 1 ml of a buffer-cyanide-methyl "Cellosolve" solution (Chibnall, Mangan, and Rees 1958). The tubes were heated in a boiling water-bath for 20 min before dilution with 50% ethanol and estimation in a Coleman Junior spectrophotometer at 570 m μ (Moore and Stein 1948). As standards, commercial samples of purified amino acids were checked and the absorbances per μ mole were compared with those obtained using the Beckman-Spinco standard mixture run through our column. The agreement was good (see Table 2) except for the two commercial samples of histidine. The average Spinco absorbances per μ mole were used to calculate our results.

(g) *Nitrogen Determinations*

These were estimated by the Kjeldahl procedure and amide nitrogen (and extraneous ammonia nitrogen) was driven off prior to each determination by evaporating freeze-dried aliquots (5 ml) of the hydrolysate in the presence of 0.1M potassium carbonate *in vacuo* over concentrated sulphuric acid (Moore and Stein 1951). There is no evidence that this treatment removes other than ammonia nitrogen (Sanger, Thompson, and Kitai 1955). It was deemed necessary to give the hydrolysates this treatment prior to nitrogen determinations because of the

difficulty in ensuring that all the urea nitrogen had been removed by the dialysis procedures.

(h) Preparative Procedures

For bulk fractionation of α -keratose the stored aqueous solution was precipitated at pH 4 with acetic acid in the presence of 0.3M potassium chloride, centrifuged lightly, and the precipitate dissolved in the 8M urea buffer. This solution was then dialysed overnight versus buffer before loading on a 2-cm dia. column of DEAE-cellulose. Elution was carried out at the rate of 20 ml/sq.cm/hr successively with 40 ml of buffer, and then buffer containing 0.1M, 0.2M, and 0.5M potassium chloride. 5-ml fractions were collected. The fractions comprising the three peaks were dialysed against water, freeze-dried, and the chromatography repeated. Finally they were dialysed successively versus water, 0.1M sodium chloride, and finally water (using a rocking dialyser and 18/32 Visking cellulose tubing) and freeze-dried for analysis.

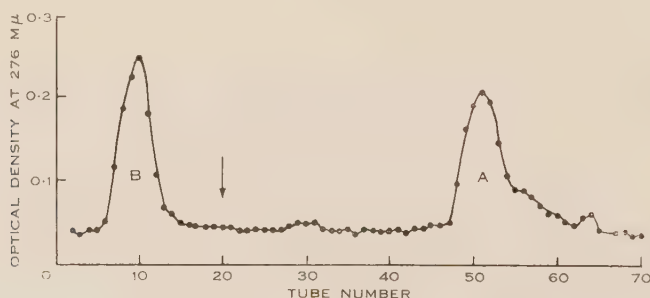


Fig. 1.—Chromatographic behaviour at 25°C of oxidized insulin (1 hr at 0°C) on DEAE-cellulose in 8M urea-Tris buffer at pH 7.4. Fraction size was approximately 1.1 ml. Protein applied in 8M urea-Tris buffer and eluted with a gradient to 0.5M potassium chloride in this buffer. Gradient reached column at tube 20.

III. RESULTS AND DISCUSSION

(a) Chromatography of Unfolded Proteins

Figure 1 shows the gradient elution curve of oxidized insulin in a buffer containing 8M urea. It is obvious that the A and B chains have separated, the more acidic glycyl chain (from absorption spectra) being retarded. It is seen that each of these peaks is itself spread out much more than would be the parent insulin peak under the same gradient (cf. Thompson and O'Donnell 1960). This might be attributable to the incomplete disaggregation of the B chain in 8M urea or to a general variation in shape amongst the molecules comprising each oxidized chain. However, it is apparent that polypeptides in an unfolded state can still be adsorbed and desorbed from a column in spite of the large number of binding sites on these molecules.

The effect of standing oxidized insulin in pH 7.4 or 8 buffer overnight before chromatography was investigated but no difference in pattern due to this standing was noted. Hence it appears that any formylation of the serine and threonine residues

(Kienhuis, Blaase, and Matze 1959; Narita 1959; Smillie and Neurath 1959) caused by formic acid has not affected the chromatographic pattern.

We have also chromatographed bovine plasma albumin after oxidation with performic acid and also after reduction and alkylation of the thiol groups. With a similar gradient to that used for the oxidized insulin a single spread-out peak was obtained in each case (which may contain more than one component). The oxidized bovine plasma albumin was considerably more spread than the reduced and alkylated material (see Fig. 2).

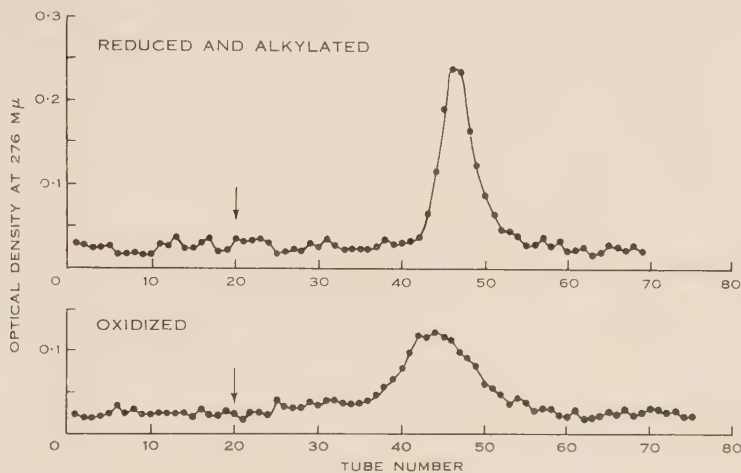


Fig. 2.—Chromatography at 25°C of oxidized, and reduced and alkylated, bovine plasma albumin on DEAE-cellulose. Protein applied in 8M urea-Tris buffer at pH 7.4 and eluted with a gradient to 0.5M potassium chloride in this buffer. Gradient reached column at tube 20. Fraction size was approximately 1.1 ml.

Figure 3(b) shows the curve obtained by gradient elution of α -keratose. The pattern recalls the curve obtained by gradient elution of γ -globulin from calcium phosphate columns (Tiselius, Hjerten, and Levin 1956) and by liquid-liquid partition chromatography (Porter 1955). These authors showed that cuts from various positions in the main peak rechromatographed in their original positions and were not unduly spread; hence the wide spreading of the original peak may be indicative of a considerable number of closely related proteins (Porter 1955). We wished to show whether the spreading in the peak of α -keratose was due (at least in part) to chemically distinct proteins and, since Tiselius, Hjerten, and Levin (1956) concluded that stepwise elution gives better resolution than gradient elution, we concentrated on the stepwise procedure; this also gives more concentrated fractions.

One stepwise curve is shown in Figure 3(a); the number of peaks obtained is arbitrary and if the increments in ionic strength were made smaller more peaks would almost certainly be obtained.

Figure 4 shows a three-step elution pattern of α -keratose and also the patterns obtained by rechromatography (after dialysis and concentration of each of the three peaks).

(b) *Interpretation of Chromatographic Data*

In the stepwise or gradient elution of proteins the interpretation of the effluent curve is not simple (cf. Boardman 1959). Apart from a few relatively small proteins (see Moore and Stein 1956) it is generally difficult to elute a protein from a column of adsorbent without considerable tailing unless the R_F is close to 1. At lower R_F 's (for a tailing zone, i.e. a substance which has a strongly curved adsorption isotherm) each change in the eluent concentration (including anomalous changes or double-fronting (Björk 1959)) can give rise to a "false" peak which may not be

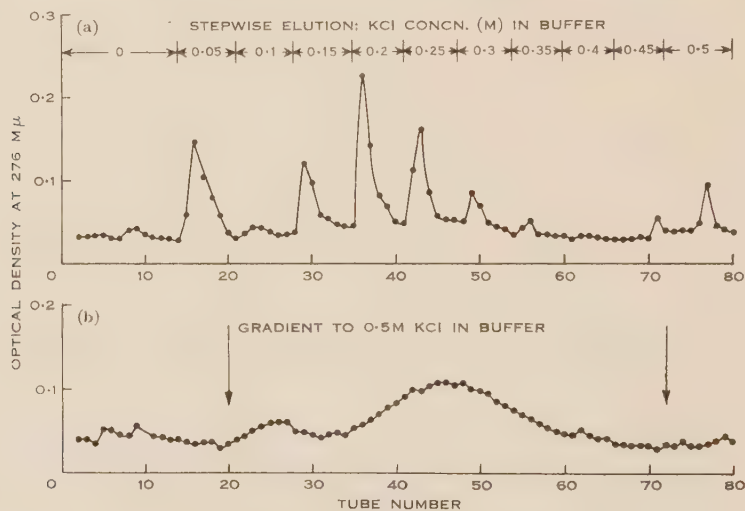


Fig. 3.—Chromatographic behaviour at 25°C of α -keratose on DEAE-cellulose in 8M urea-Tris buffer at pH 7.4. Fraction size was approximately 1.1 ml. (a) Stepwise elution with increasing concentrations of potassium chloride in the buffer; (b) gradient elution to 0.5M potassium chloride in the buffer.

indicative of chemically differing components. It might be expected that gradient elution would be preferable to stepwise elution in such cases but Tiselius, Hjerten, and Levin (1956) have pointed out that in this case the resolution into separate peaks is never as good as in stepwise elution.

With stepwise elution heterogeneity may be wholly or partly masked since several discrete chemical components may be eluted as a single peak particularly if the increments in ionic strength of the eluent are large.

Despite the difficulties of interpretation gradient or stepwise elution can demonstrate heterogeneity present in a solution of protein. Before claiming definite separation of components, however, each separated fraction should be rechromatographed to show that it runs in its original position, i.e. its behaviour when separated

is the same as when in the mixture. Moreover, the characterization by some specific property (e.g. biological activity or amino acid composition) of the material in the various peaks makes certain whether or not a fractionation has occurred (Moore and Stein 1956; Tiselius, Hjerten, and Levin 1956).

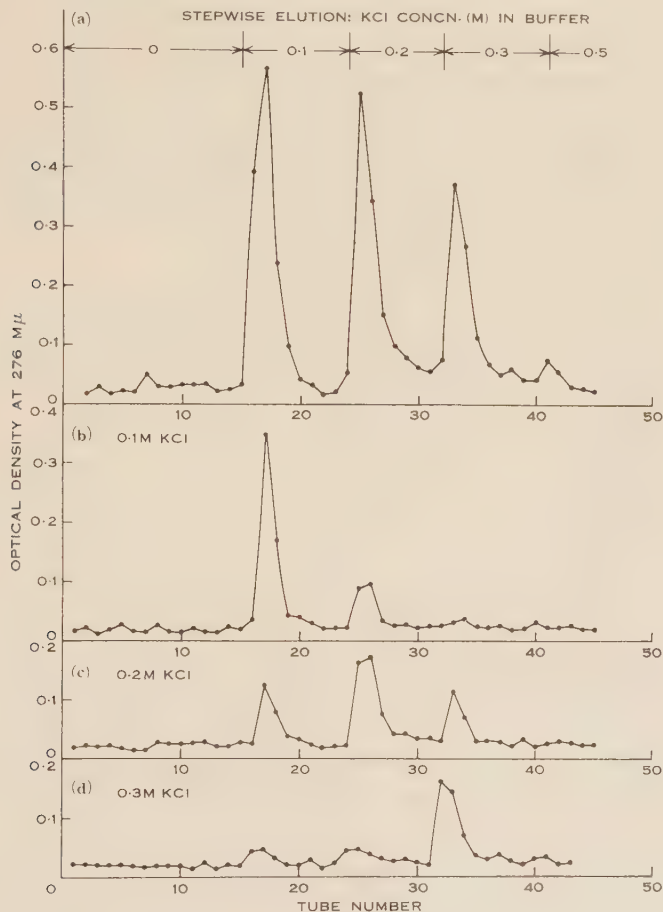


Fig. 4.—Chromatographic behaviour at 25°C of α -keratose on DEAE-cellulose in stepwise elution (a) and rechromatography on the same column of fractions obtained (b)–(d). Protein applied in 8M urea-Tris buffer at pH 7.4 and eluted with 0, 0.1, 0.2, 0.3, and 0.5M potassium chloride in this buffer. Fraction size was approximately 1.1 ml.

Although each cut of our α -keratose patterns (Fig. 4) is not pure, since chromatography by one-step elution is an inefficient process, it is seen there is a considerable enrichment of each component in the rechromatography and this alone points to the fact that a real separation of differing components has been achieved (cf. Boman and Westlund 1956).

(c) *Chemical Characterization of Fractions from α -Keratose*

Ultracentrifugal studies on α -keratose have not yielded any definite evidence on heterogeneity. This is due to the complication introduced by aggregation of the protein (O'Donnell and Woods 1956*a*, 1956*b*). Similarly aggregation makes it impossible to interpret the electrophoretic patterns or to state definitely that electrophoretic heterogeneity is present (O'Donnell and Woods 1956*a*). The electrophoretic pattern is predominantly a single peak. End-group analyses on α -keratose

TABLE I
AMINO ACID CONTENTS OF ACID HYDROLYSATES OF THE THREE
FRACTIONS OF α -KERATOSE ISOLATED BY STEPWISE ELUTION
FROM A COLUMN OF DEAE-CELLULOSE

Values in g amino acid nitrogen per 100 g protein nitrogen
(excluding amide nitrogen)

Amino Acid	0.1M KCl Fraction	0.2M KCl Fraction	0.5M KCl Fraction
Cysteic acid	4.70	4.80	6.19
Lysine	6.26	5.53	4.56
Histidine	1.84	1.70	1.94
Arginine	22.8	21.4	20.2
Ammonia	9.45	10.7	10.2
Tryptophan plus oxidized tryptophan*	1.18	1.0	1.55

* These values are not g amino acid nitrogen per 100 g protein nitrogen but are the ratio of absorbances per unit of nitrogen in the hydrolysate, with the 0.2M KCl fraction as standard for the peak eluted between the phenylalanine plus tyrosine and the lysine peak. This is the normal position of tryptophan (Moore, Spackman, and Stein 1958). Our peak had more than one component and no molar absorbance factor could be assumed.

fractions reveal the presence of small amounts of a variety of end-groups, also present in wool, and point to the fact that there are chemically different components in α -keratose (Alexander and Smith 1956) even if it is assumed that masked terminal groups or cyclic molecules are predominant (Thompson 1959).

In order to determine whether the components comprising the elution curve of α -keratose differed radically in their composition or whether only minor differences such as amide content were responsible for the separation an arbitrary fractionation (again by three-step elution) with salt concentrations of 0.1M, 0.2M, and 0.5M potassium chloride was carried out. These fractions were approximately each one-third of the protein put on the column. Each peak was further purified by rechromatography and the isolated fractions were analysed for cysteic acid and the basic amino acids lysine, histidine, and arginine. The hydrolysates of the proteins were prepared simultaneously so that losses during hydrolysis should be identical. The results are shown in Table I. It is seen that there are large differences in amino

acid composition between the peaks eluted with buffer containing 0.1M and 0.5M potassium chloride, particularly in cysteic acid and lysine. The differences between the peaks eluted with buffer containing 0.1M and 0.2M salt are very much less pronounced. The differences in ammonia values between the 0.1M and 0.2M potassium chloride peaks may be real but no definite conclusions can be drawn due to the possibility that all of the urea has not been removed from one of the fractions.

The ultraviolet absorption curves of the three hydrolysates (of equal protein concentration) in 2N hydrochloric acid are shown in Figure 5; it is seen that the curves for the 0.1M and 0.2M potassium chloride peaks are similar but there is a pronounced difference in the height of the peaks at 270–280 m μ between those for 0.1 or 0.2M and 0.5M potassium chloride.

TABLE 2
NINHYDRIN COLOUR ABSORBANCE VALUES

Ninhydrin colour developed by procedure given in text and measured in matched tubes in a Coleman Junior spectrophotometer at 570 m μ (Moore and Stein 1948). Values given are absorbances per μ mole amino acid

Amino Acid	NH ₄ Cl	Sample 1*	Sample 2†	Sample 3‡
Ammonia	3.62 } 3.82 } 3.72			3.68 } 4.08 } 3.88
Arginine		3.94	3.86	4.00 } 3.90 } 3.95
Histidine		4.16	3.59	3.88 } 3.98 } 3.93
Lysine		4.25	4.34	4.32 } 4.36 } 4.34

* From California Corporation for Biochemical Research, Los Angeles, California.

† From Nutritional Biochemicals Corporation, Cleveland, Ohio.

‡ Beckman–Spinco standard mixture of amino acids after separation on "Dowex-50".

For the concentrations of amino acids being examined here (except histidine) Moore and Stein (1948, 1954) and Moore, Spackman, and Stein (1958) find that recoveries from standard mixtures of amino acids are $100 \pm 3\%$ of theory so that a difference of 6% between duplicate runs is possible. Duplicate runs on a standard Spinco mixture of amino acids showed the variation for lysine, histidine, and arginine to be less than 3% (see Table 2). Hence there are probably real differences in amino acid content between the peaks eluted with buffer containing 0.1M and 0.2M potassium chloride. If the spread pattern obtained by gradient elution is due to a considerable number of closely related proteins it might be expected that the differences between some successive fractions would be quite small and may not involve the amino acids determined here.

It is possible that amide differences alone contribute to the spreading during gradient elution of the original α -keratose. Furthermore it seems probable, in view of the oxidized insulin pattern, that even in 8M urea differences in states of aggregation or molecular shape or both could contribute to the spread.

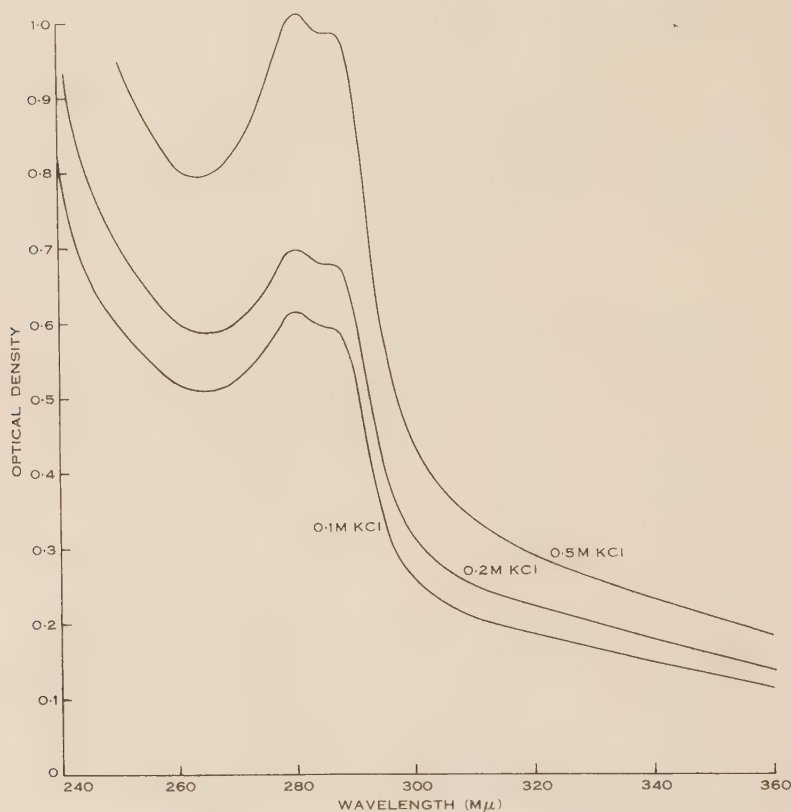


Fig. 5.—Ultraviolet absorption spectra of acid hydrolysate of the three fractions of α -keratose. Concentration was approximately 0.8 mg per ml in 2N HCl.

(d) γ -Keratose

γ -keratose, the high-sulphur, low molecular weight fraction from oxidized wool, when examined by gradient elution gave a similar type of pattern to α -keratose with maxima at approximately the same elution volumes (see Fig. 6(b)). It could also be divided into several peaks by stepwise elution (Fig. 6(a)). Since α - and γ -keratose differ markedly in amino acid composition (Gillespie *et al.* 1960) the increased acidity of γ -keratose which would tend to increase the elution volume over that exhibited by α -keratose must be counterbalanced by other factors such as smaller molecular size and shape. It should be noted that preparations of γ -keratose are electrophoretically heterogeneous (Woods, unpublished data) in agree-

ment with the heterogeneity of the high-sulphur protein from reduced and alkylated wool (Gillespie 1959).

(e) *Is there a Major Homogeneous Protein in Wool?*

The question arises as to the whereabouts of the repeat unit in wool which is responsible for the striking regularity in the X-ray diffraction pattern. If it is to come from a major homogeneous protein component then the present work yields no positive evidence that such a component exists; furthermore, end-group analysis on intact wool (Middlebrook 1951) and extracted wool proteins (Alexander and

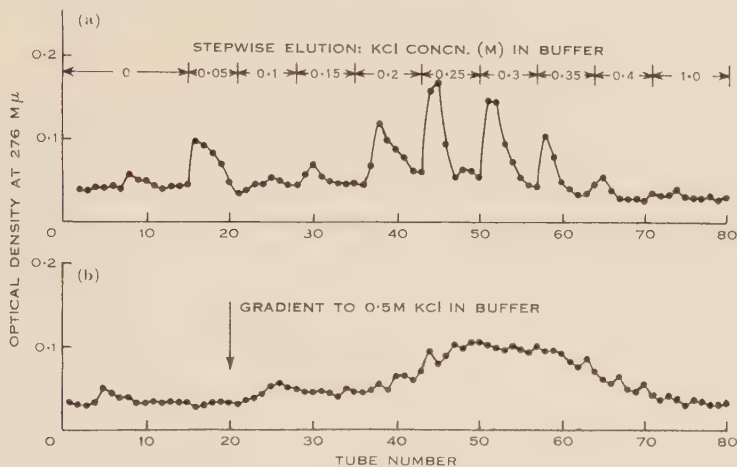


Fig. 6.—Chromatographic behaviour at 25°C of γ -keratose on DEAE-cellulose in 8M urea-Tris buffer at pH 7.4. Fraction size was approximately 1.1 ml. (a) Stepwise elution with increasing concentrations of potassium chloride in the buffer; (b) gradient elution to 0.5M potassium chloride in the buffer.

Smith 1956; Thompson 1957) reveal a large variety of terminal amino acids each in small amount. One possible interpretation of these facts is that there exists in wool a family (or families) of closely related proteins with different terminal residues. This may be the case even if masked terminal groups or cyclic structures are present and the detected terminal amino acids represent impurities. The possibility of complications arising from the degradative procedures used in the isolation of soluble proteins from wool must always be kept in mind. Oxidation with performic acid is known to modify tryptophan but this does not occur when preparing the *S*-sulphokerateines (Swan 1959); yet the gradient elution diagrams of proteins prepared by these two methods* are similar (the elution of non-acid-precipitable (pH 4) *S*-sulphokerateine required a much steeper gradient than did γ -keratose to elute it in a similar volume).

* The acid-precipitable and non-acid-precipitable *S*-sulphokerateines were prepared in a similar manner to the α - and γ -keratoses from the extract of wool using 8M urea, 0.1M sodium sulphite, and 0.05M sodium tetrathionate (cf. Bailey and Cole 1959). 50% of the wool dissolved in 48–72 hr at room temperature.

An alternative viewpoint could arise from the postulation of multiple-stranded cables of α -helixes (Crick 1952; Pauling and Corey 1953) in the crystalline regions of keratin. The repeat unit could then be visualized as consisting of several distinct protein components interwoven with each other, the percentage of any "pure" component being small.

Because of the difficulties in interpreting the elution patterns during stepwise or gradient elution it is not possible to conclude how many chemically different components there may be in α -keratose, but we feel the stepwise elution pattern (Fig. 3(a)) suggests there may well be a considerable number of such components even though some or all of these could be derived from a parent protein.

(f) *Heterogeneity of Wool Proteins*

The yields and properties of extracted wool proteins isolated by various methods have recently been summarized (Gillespie *et al.* 1960). It would be expected that α -keratose, which can amount to 53–60% of the wool fibre, would be heterogeneous, in line with the multicomponent nature of wool proteins extracted by reduction and alkylation; here the major electrophoretic component is estimated to be 41% of the wool (Gillespie and Lennox 1955) and possibly consists of two components at least (Gillespie 1960). Thus the large difference in components eluted with 0.1 or 0.2M and 0.5M KCl probably correspond to macroheterogeneity. However, the differences between components eluted with 0.1 and 0.2M KCl according to the properties measured here are very slight and fractionation by smaller increments of salt concentration (e.g. Fig. 3(a)) would give fractions which might be difficult to distinguish by even analysis for all of the amino acids. This chromatographic heterogeneity may thus be evidence of microheterogeneity in the extracted wool protein.

When proteins are isolated from tissues and are purified there are many cases where these purified proteins can be further separated chromatographically to give components of identical biological activity (see Colvin, Smith, and Cook 1954). Similar separations can often be achieved by zone electrophoresis and this suggests that the main difference is one of charge and possibly shape. Even with cautious extraction procedures, ribonuclease contains at least two components (Martin and Porter 1951; Hirs, Moore, and Stein 1953; Åquist and Anfinsen 1959), one of which is lacking the *N*-terminal lysine (King, Yphantis, and Craig 1960). Prolactin can be separated into several forms (Cole and Li 1959; Cole and Mendiola 1960). Carsten and Pierce (1960) likewise have demonstrated the presence of several components in beef thyrotropic hormone. More recently Press, Porter, and Cebra (1960) demonstrated the presence of at least 10 molecular forms in cathepsin D by chromatography on DEAE- and carboxymethylcellulose and starch-gel electrophoresis. These authors endeavoured to show that autolysis was not responsible for this complexity. There are many other less well-characterized examples (for summary see Markert and Møller 1959; Miller, Blum, and Hamilton 1960).

Thus the presence of many closely related components in wool would not be surprising particularly as the fibre must undergo many changes in passing from its site of synthesis to the keratinized fibre.

It is currently believed that the material responsible for the regular X-ray diagram of wool is part of the microfibrillar protein (see Fraser, MacRae, and Rogers 1959) and that this protein gives rise to α -keratose on oxidation and subsequent alkaline extraction of wool (see Alexander and Smith 1956; Birbeck and Mercer 1957; Rogers 1959). Proteins derived from this material might be expected to be less heterogeneous than those derived from the matrix, i.e. γ -keratose.

If there are regular repeating lengths of polypeptide chain in a family of closely related proteins such as we have envisaged for the crystalline region, a study of the peptides produced by limited degradation of α -keratose, e.g. by trypsin, and "fingerprinting" should reveal these similarities. Work along these lines is in progress.

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PREPARATION OF PURE PROTEINS FROM HOG THYROID GLANDS BY COLUMN CHROMATOGRAPHY ON DIETHYLAMINOETHYL- CELLULOSE

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Summary

Saline extracts of hog thyroid glands were chromatographed on columns of diethylaminoethyl (DEAE)-cellulose using gradients of pH and salt concentration. Two major peaks of protein emerged from these columns. When rechromatographed under identical conditions, part of the first peak to emerge from the column redistributed itself to give two peaks which appeared in positions corresponding to those of the two peaks eluted from the first chromatographic run.

Examination of the ultracentrifugal components present in the eluted protein fractions and their comparison with the components present in the original saline extracts indicated that some resolution of the ultracentrifugal components had taken place during chromatography on DEAE-cellulose. After further purification by a run in the separation cell, about 96% of the sedimentary material was located in the peak with $S_{20,w} = 18$. Most such runs gave even higher purity.

Quantitative paper chromatography of the iodoamino acids of an enzymic hydrolysate of protein from the first peak and from three parts of a broad second peak eluted from a column of DEAE-cellulose indicated that the ratio of iodotyrosines to iodothyronines was 1.9, 3.7, 4.2, and 8.8, respectively.

I. INTRODUCTION

The first extensive study of methods for the preparation of thyroglobulin from thyroid glands of a number of species was described by Derrien, Michel, and Roche (1948). By fractional salting-out of the proteins present in saline extracts of sliced glands they obtained thyroglobulins which were homogeneous in the ultracentrifuge and during electrophoresis according to Tiselius (Derrien, Michel, and Roche 1948; Derrien *et al.* 1949). Crude thyroid extracts are known to contain three major and two minor ultracentrifugal components‡ (Shulman, Rose, and Witebsky 1955). Thyroglobulin prepared by the method of Derrien, Michel, and Roche (1948) exhibits three breaks in the salting-out curve which suggest that it comprises three molecular entities differing slightly in their solubility properties (Derrien, Michel, and Roche 1948). This concept is also supported by the finding of three precipitin bands by the method of Ouchterlony (1948) when human thyroglobulin and an antiserum prepared in a rabbit were allowed to diffuse together (Easty, Slater, and Stanley 1958).

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‡ In this paper the term "ultracentrifugal component" refers to material sedimenting in a particular peak during ultracentrifugal analysis. It is realized that such a "component" need not be homogeneous when subjected to other methods of fractionation.

A detailed description of salting-out methods for purification of the proteins of thyroid extract is being published from this Laboratory (Shulman and Witebsky 1961).

Sober *et al.* (1956) described the fractionation of serum proteins on columns of diethylaminoethyl (DEAE)-cellulose prepared according to Peterson and Sober (1956) and this procedure has now been applied to a study of the proteins present in saline extracts of hog thyroid glands in the hope of developing a simple and rapid method for the preparation of these proteins in a pure form. A preliminary report has been published (Shulman and Witebsky 1960).

II. MATERIALS AND METHODS

Saline extracts were prepared as described by Shulman, Rose, and Witebsky (1955) from hog thyroid glands which had been stored in the frozen state. Protein concentrations were measured by the extinction at $280\text{ m}\mu$. Because of the variable composition of the buffers used for the elution of the proteins, the extinctions of the effluent fractions were read against a blank of distilled water: when samples of the fractions had to be diluted, 0.15M NaCl was used as diluent and the same solvent was used in the reference cell of the spectrophotometer. Additional readings of the optical densities of saline extracts and eluted fractions were taken at $407\text{ m}\mu$ in order to record the intensity of the red-orange colour. Extracts were chromatographed immediately or were freeze-dried and stored at $2-4^{\circ}\text{C}$.

DEAE-cellulose, type 20, lot 1023, was obtained from the Brown Company, Berlin, New Hampshire, U.S.A. It was employed without pretreatment other than adjustment of the pH to the starting value. Used DEAE-cellulose was first regenerated by washing with a large excess of 1N NaOH followed by exhaustive washing with distilled water (Peterson and Sober 1956). DEAE-cellulose (29 g) was suspended in distilled water (1 l.) and the pH brought to $7.0-7.2$ by gradual addition of 0.5M NaH_2PO_4 (about 35 ml) to the suspension with vigorous stirring. The column (2.5 cm internal diameter) was filled with 0.1M phosphate buffer, pH 6.96 , and the suspension of DEAE-cellulose added gradually, permitting larger particles to settle on the fritted glass disk at the bottom of the column. The column was washed with 0.1M phosphate buffer (pH 6.96 , $0.5-1.0\text{ l.}$), then with an equal volume of starting buffer (0.005M phosphate buffer, pH 7.2). It was compressed with N_2 (10 lb/in^2 , $20-60\text{ sec}$). Saline extracts of hog thyroid glands (fresh or reconstituted from freeze-dried material) were dialysed with stirring against several changes of starting buffer. Initially the elution schedule was that used for human serum proteins by Sober *et al.* (1956, p. 759, fig. 3). Our subsequent modifications are described in the legends to Figures 2 and 4. Fractions of 6.1 ml were collected mechanically by a volumetric type of collector. The flow rates did not exceed 1 ml/min .

Protein solutions were concentrated by dialysis with stirring against concentrated solutions of polyvinylpyrrolidone. The polymer solutions had been exhaustively dialysed against 0.15M NaCl before use in order to free them of contaminants of low molecular weight. Alternatively, proteins were concentrated by freeze-drying and resolution.

Enzymic Hydrolysis.—Samples of proteins were subjected to enzymic hydrolysis in sealed tubes for 48 hr in the presence of toluene. A mixture of crystalline trypsin (14.9 mg), crude trypsin (4.9 mg), crystalline chymotrypsin (5.1 mg), and of a suspension of carboxypeptidase (0.5 ml) was used per 100 mg thyroidal protein in a 2-amino-2-hydroxymethylpropane-1,3-diol buffer, pH 8. The pH of the hydrolysates was adjusted to about 1.3 by gradual addition of 1N HCl and the iodoamino acids were extracted with three successive portions of *n*-butanol (25 ml), previously saturated with dilute HCl (pH 1.15), the pooled *n*-butanolic phases were made alkaline

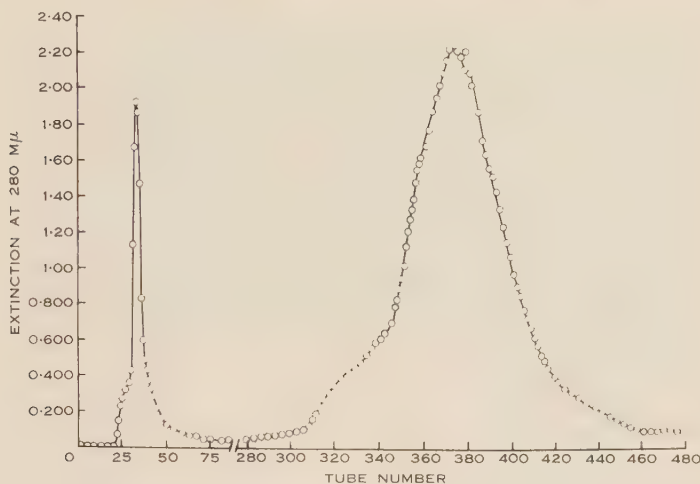


Fig. 1.—Elution pattern of thyroidal proteins (1.56 g, crude saline extract) applied to a column of DEAE-cellulose (column 3, 29 g, pH 7.1) and developed with the following buffers: 0.005M phosphate buffer (pH 7.2, 382 ml); 250 ml of the same buffer in mixing flask, gradient to 0.02M phosphate buffer (pH 6.0, 205 ml); gradient to 0.05M NaH_2PO_4 (pH 4.53, 480 ml); gradient to 0.05M NaH_2PO_4 –0.02M NaCl (365 ml); gradient to 0.05M NaH_2PO_4 –0.05M NaCl (pH 4.48, 750 ml); gradient to 0.05M NaH_2PO_4 –0.1M NaCl (pH 4.43, 1150 ml). Volume of fractions: 6.1 ml.

by addition of 2N NH_4OH (15 ml) and the iodoamino acids stabilized by addition of thiouracil (1.33 mg in 1 ml water). The solutions were reduced to dryness and iodoamino acids determined by the method of Mandl and Block (1959).

Crude saline extracts of thyroid glands as well as protein fractions eluted from the columns of DEAE-cellulose were examined in the ultracentrifuge (Spinco, model E) either with or without prior adjustment of the solvent by dialysis against 0.15M NaCl. Concentrations of about 0.7% were found to be optimal for most of the fractions examined. Higher concentrations were used at times to detect impurities in isolated components.

III. RESULTS

The extinctions at 280 $\text{m}\mu$ of the fractions collected from the column (column 3) are plotted against the number of the tube in Figure 1. This column was developed with the buffers described by Sober *et al.* (1956, p. 759, fig. 3). The protein emerged from the column in two major peaks, the recovery being 33% of the protein applied to the column.

The fractionation of thyroïdal proteins on a new column (column 8) run with a modified system of buffers is illustrated in Figure 2; the buffers used are described in the legend. Again two major peaks of protein (*A* and *D*) were eluted from the column; 70% of the protein applied to this column was recovered in peaks *A* and *D*, an additional 2% in minor peaks. Part of the material from peak *A* of column 8 was rechromatographed on a fresh column (column 9) under identical conditions. The elution pattern is shown in Figure 2. Protein again appeared in two major peaks corresponding in position to peaks *A* and *D* of column 8. Of the protein applied to

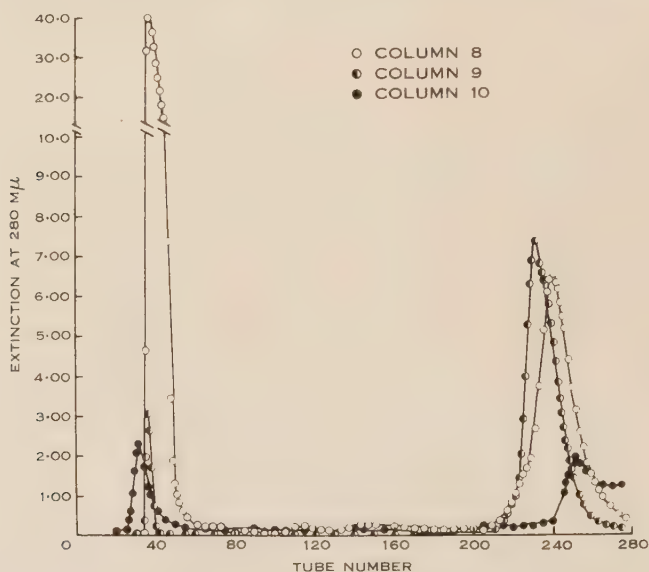


Fig. 2.—Elution patterns of thyroïdal proteins applied to column 8 (3.96 g, crude saline extract), column 9 (1.21 g, peak *A* from column 8), and column 10 (67.5 mg, peak *A* from column 9) and developed with the following buffers: Columns 8 and 9: 0.005M phosphate buffer (pH 7.2, 350 ml); 250 ml of the same buffer in mixing flask, gradient to 0.05M NaH_2PO_4 (pH 4.53, 200 ml); gradient to 0.05M NaH_2PO_4 –0.05M NaCl (pH 4.48, 300 ml); gradient to 0.05M NaH_2PO_4 –0.1M NaCl (pH 4.43, 300 ml); gradient to 0.1M NaH_2PO_4 –0.2M NaCl (pH 4.07, 950 ml). Column 10: Identical, except that the gradient to 0.05M NaH_2PO_4 was set up with 300 ml of 0.05M NaH_2PO_4 , retarding the emergence of the second peak. Volume of fractions: 6.1 ml. Each column contained 29 g. of DEAE-cellulose (pH near 7). Extinctions have been multiplied by 20 for column 10.

column 9, 83% was recovered in peaks *A* and *D*. Part of the material from peak *A* of column 9 was re-run on a fresh column (column 10). The elution pattern is reproduced in Figure 2. The extinction values have been multiplied by 20. Again two peaks emerged from the column which corresponded in position to peaks *A* and *D* of column 8.

Examination of the proteins eluted in peak *A* of column 8 in the ultracentrifuge revealed that some fractionation of the ultracentrifugal components present in the original saline extract had taken place in the column, resulting in an almost complete absence of the slow component and the presence of only the 18-S and 29-S

components in this fraction. Four ultracentrifugal components were present in peak *D* of column 8. In a number of subsidiary experiments, material from peak *A* of column 8 was ultracentrifuged in a separation cell and an 18-*S* component of a high degree of purity isolated from the upper compartment. Although a small 11-*S* peak appeared in this particular fraction (run 2915 in Table 1), other samples obtained in exactly this way showed no detectable 11-*S* boundary, and thus appeared to contain close to 100% of the sedimenting material in the peak with $S_{20,w} = 18$. An example of such an ultracentrifugal appearance (run 2924) is shown in Figure 3 (c), where it

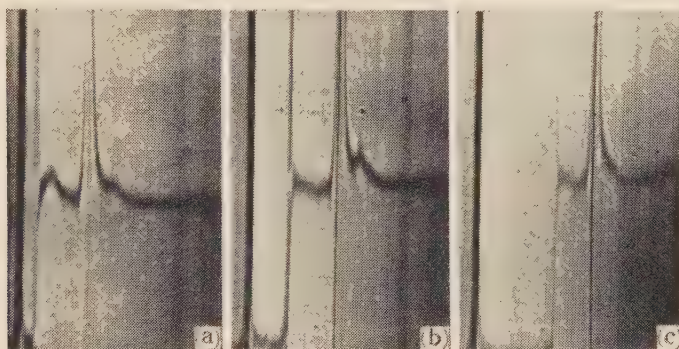


Fig. 3.—Ultracentrifugal patterns of (a) saline extract of hog thyroid tissue, run 2902; (b) peak *A* material from column 8, run 2909; (c) separation cell top component obtained from peak *A* of column 8, run 2924. Protein concentration 0.7% in each. Phase-plate angle 30°. Photographs were taken 8 min after rotor reached speed of 59,600 r.p.m.

can be compared with that of saline extract (run 2902) in Figure 3 (a), and peak *A* before use of the separation cell (run 2909) in Figure 3 (b). The $S_{20,w}$ values and percentage compositions of the proteins present in the original saline extracts and of some of the fractions are shown in Table 1.

In an additional column we modified not only the system of buffers but also the initial pH of the DEAE-cellulose and of the starting buffer. The elution pattern of this column (column 11) is shown in Figure 4; the buffers used are described in the legend. Two major peaks of protein emerged from this column in positions corresponding to peaks *A* and *D* of column 8, with an additional intermediate protein peak which coincided with a peak of the red-orange pigment (absorption maximum 407 $m\mu$) of the thyroid extract. Although the final buffer used with column 11 was 0.25M NaH_2PO_4 -2M NaCl, further elution of this column with 0.1N NaOH* led to the emergence of additional protein equivalent to about 11% of the protein applied.

Samples of material corresponding to peak *A* (column 8) and from three parts of a broad peak corresponding to peak *D* (column 8) were obtained from another chromatographic fractionation and were hydrolysed enzymically. The percentages of iodoamino acids in the four samples are listed in Table 2.

* We are indebted to Mr. J. Armenia for continuing the elution of column 11 with 0.1N NaOH.

IV. DISCUSSION

The appearance of two major protein fractions during the development of the DEAE-cellulose columns suggested that the thyroid extracts contained two types of protein differing in their ionizing groups. The rapid appearance of the first protein

TABLE I
 $S_{20,w}$ VALUES AND APPROXIMATE PERCENTAGE COMPOSITIONS OF THE ULTRACENTRIFUGAL COMPONENTS PRESENT IN THE ORIGINAL SALINE EXTRACTS OF HOG THYROIDS AND OF FRACTIONS OBTAINED FROM THESE BY COLUMN CHROMATOGRAPHY ON DEAE-CELLULOSE

Run No.	Material	Concn. (%)	$S_{20,w}$	% of Total
2588	Saline extract used for column 3	0.50	28.4	10.0
			18.0	78.4
			4.3	11.5
2590	Column 3, pool B (peak A)	0.45	18.8	84.1
			c.4	15.9
2591	Column 3, pool D (peak D)	0.79	*	5.1
			17.7	74.8
			12.6	4.2
2592	Column 3, pool C (peak D)	0.35	0.68	15.9
			17.7	68.9
			12.2	3.9
2593	Column 3, pool E (peak D)	0.59	4.5	27.2
			*	20.1
			18.3	73.5
2902	Saline extract used for column 8	0.70	12.2	6.4
			25.7	11.3
			18.2	73.8
2909	Column 8, peak A	0.70	4.7	14.9
			29.8	10.6
			18.1	89.4
2915	Column 8, peak A, top of separation cell run	1.00	15.6	95.7
			11.1	4.3
2933	Column 8, peak D	0.76	29.1	6.0
			17.9	82.7
			11.4	3.0
2934	Column 8, peak E	0.76	5.0	8.2
			40.3	45.3
			18.5	39.0
2936	Column 8, peak C	0.68	3.9	11.1
			*	4.5
			8.5	38.9
2952	Saline extract, not used for chromatography	0.80	7.3	61.0
			27.9	7.8
			17.9	82.2
2953	Column 9, tube 231	0.73	4.0	10.0
			31.2	10.5
			17.3	85.3
			13.1	3.0
			*	1.2

TABLE 1 (Continued)

Run No.	Material	Concn. (%)	$S_{20,w}$	% of Total
2954	Column 9, tube 236	0.64	40.6 19.0 15.6 11.7	6.7 89.2 2.7 1.4
2958	Column 9, peak A	0.73	25.1 19.7 13.9 6.9 2.6 3.0	10.7 72.3 2.6 7.4 7.0
2989	Column 10, peak A	0.56	6.5 3.3	37.0 63.0
2992	Column 10, peak D	0.8	17.4 11.5	89.3 10.7
2997	Column 10, peak A	0.56	6.8 3.4	41.2 58.8
3012	Column 11, tube 34 (phosphate)	2.52	18.3 12.6 11.5 3.0	† † † †
3013	Column 11, tube 34	0.77	20.1 14.4 11.7	† † †
3015	Column 11, tube 34 (phosphate)	2.52	* 11.6 7.3 2.2	† † † †
3016	Column 11, tube 245 (phosphate)	1.23	19.1 16.0 12.5 4.1	† † † †

* Insufficient points to determine sedimentation rate.

† Concentration too high to determine percentages.

peak (peak A) after the start of development raised the possibility that this might be a "breakthrough" peak due to overloading of the column. The redistribution of material from peak A of column 8 when re-run under identical conditions on column 9 seemed to confirm this hypothesis, but the redistribution of protein observed with column 10 suggested that at least part of the protein invariably appeared in the position of peak A, since the possibility of overloading no longer existed in the running of columns 9 and 10. Column 11, run under such conditions of starting pH so as to increase the capacity of the DEAE-cellulose for proteins, nevertheless gave a major protein peak in the position of peak A of column 8. The exact nature of the transition which is responsible for the redistribution of protein during successive

chromatographic runs is not known. It is apparent that under certain conditions a resolution of the ultracentrifugal components constituting the thyroïdal proteins may be possible. Examination of the data in Table 1 shows that there is reasonably good agreement between the rates and compositions of three saline extracts (runs 2588, 2902, and 2952). Two minor peaks (*E* and *C*) from column 8 (runs 2934 and 2936) contained high proportions, respectively, of a fast component with $S_{20,w} = 40.3$ which may or may not be identical with component I of Shulman, Rôse, and Witebsky (1955) and of a slow component ($S_{20,w} = 7.3$) which is probably component IV of

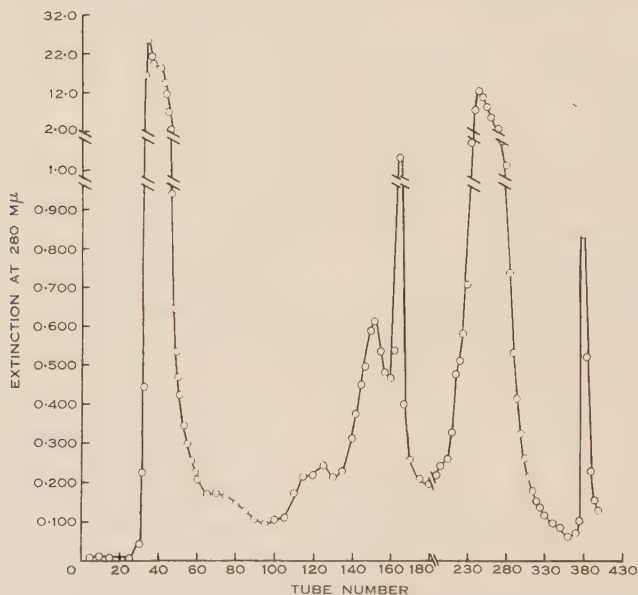


Fig. 4.—Elution pattern of thyroïdal proteins (4.40 g, crude saline extract) applied to a column of DEAE-cellulose (column 11, 29 g, pH near 8) and developed with the following buffers: 0.005M phosphate buffer (pH 8.04, 400 ml); 250 ml of the same buffer in mixing flask, gradient to 0.05M NaH_2PO_4 (190 ml); gradient to 0.05M NaH_2PO_4 –0.1M NaCl (333 ml); gradient to 0.1M NaH_2PO_4 –0.2M NaCl (825 ml); gradient to 0.25M NaH_2PO_4 –2.0M NaCl (500 ml). Volume of fractions: 6.1 ml.

Shulman, Rose, and Witebsky (1955). The additional component ($S_{20,w} = 8.5$) of column 8, peak *C* (run 2936), may be a very minor component that does not occur in adequate concentration to be detected in the original saline extract, or it may even be non-thyroidal.

Examination of the iodoamino acids present in the peak *A* and in three positions of peak *D* suggested that there was some resolution of the iodoproteins depending on the nature of their constituent iodoamino acids. Proteins which emerged from the column in peak *A* had a higher ratio of iodothyronines to iodotyrosines than those emerging in the most distant (D_3) of three parts of peak *D*, and the ratios for the two intermediate fractions (D_1 and D_2) were intermediate between those of the proteins present in the extremes (*A* and D_3). The existence in thyroglobulin of proteins, similar in most respects, but differing markedly in their relative contents of iodo-

tyrosines and iodothyronines was already suggested by Ingbar, Askonas, and Work (1959), but these authors did not provide direct evidence for their existence. It should be pointed out, however, that our results are preliminary and require confirmation.

While this work was in progress, Ingbar, Askonas, and Work (1959) described the chromatography on DEAE-cellulose of ovine thyroglobulin prepared by the method of Derrien, Michel, and Roche (1948). They observed a peak corresponding to the peak *A* observed in our experiments, but it is possible that they failed to continue the development of the column long enough for a second peak to emerge. As these authors supply no data concerning the amount of protein applied to and

TABLE 2
DISTRIBUTION (% OF TOTAL) BETWEEN THE VARIOUS CONSTITUENTS OF
THE IODINE PRESENT IN FOUR ENZYMIC DIGESTS OF IODOPROTEIN
FRACTIONS ELUTED DURING THE CHROMATOGRAPHY OF CRUDE THYROID
EXTRACT ON DEAE-CELLULOSE

Iodoprotein Fraction	Enzymic Digest			
	<i>A</i>	<i>D</i> ₁	<i>D</i> ₂	<i>D</i> ₃
Origin (unknown)	10	15	12	16
3,5-Diiodotyrosine	41	36	42	38
3-Iodotyrosine	13	23	25	32
Iodide	9	10	4	6
Thyroxine	26	15	15	8
3,5,3'-Triiodothyronine	2	1	1	Trace
Total iodine in sample (μg)	258	179	330	463
Ratio* of $\frac{\text{iodotyrosines}}{\text{iodothyronines}}$	1.9	3.7	4.2	8.8

* For rat and human thyroglobulins the ratio usually found is 9 : 1 or 8 : 1 (R. J. Block, personal communication, 1960).

recovered from their columns, a strict comparison between their and our findings is impossible. It is also likely that they were using DEAE-cellulose of rather high pH, as it is virtually impossible to adjust the pH of freshly activated DEAE-cellulose to 6.8 by washing it with 0.01M phosphate buffer of that pH. Furthermore, the ultracentrifugal data reported by Ingbar, Askonas, and Work (1959) cannot be compared with ours, as there is no indication of the concentrations at which the solutions were run in the ultracentrifuge.

V. ACKNOWLEDGMENTS

This investigation was supported in part by a research grant, C-3737, from the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service, Bethesda 14, Maryland. One of us (S.S.) gratefully acknowledges support through a Senior Research Fellowship (SF-118) from the U.S. Public Health Service.

We should like to express our appreciation to Professor E. Witebsky for his constant support and encouragement of this work. We are greatly indebted to Dr. R. J. Block, Boyce Thompson Institute for Plant Research, Yonkers, New York, for carrying out the quantitative analyses of iodoamino acids. We would also like to thank Mr. I. Kawai for doing the ultracentrifuge runs.

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SHORT COMMUNICATIONS

ESTIMATES OF CORTICAL DIFFERENTIATION IN NORMAL AND "DOGGY" MERINO WOOLS*

By G. JONES†

Ahmad and Lang (1957) reported a comparison of ortho- and paracortical proportions in normal and "doggy" Merino wools. The doggy samples had been selected by wool appraisers in several Australian States as characteristic examples of wools regarded in their States as markedly doggy. The normal wools with which they were compared were well-crimped wools of Australian origin, not necessarily from the same source as the doggy wools.

Further work has been conducted at this Institute in attempts to characterize doggy wools (Glynn, Lang, and Wardle 1960) and to discover differences in manufacture of doggy as compared with normal wools (Lang and Sweetten 1960). In these two studies, attention has been confined to normal and doggy wools from single flocks. In these wools the doggy samples may be regarded as intermediate between the normal and those doggy wools studied by Ahmad and Lang.

Glynn, Lang, and Wardle (1960) reported the types of segmentation of the ortho- and paracortices, and that the onset of "dogginess" was accompanied by a tendency to a change of type of segmentation. A preliminary reference to the present author's work on the percentage differentiation on the same wools was made. This work is reported herein.

Experimental

(i) *Wools Used*.—The samples of normal and doggy wools used were those designated as groups B and C by Glynn, Lang, and Wardle (1960):

Group B: Numbered R49 N and D, R50 N and D, R51 N and D. These were three of the pairs of manufacturing research bulks, each pair being normal and doggy wools from a single flock: R49 and R50 were Merino wool of 64's quality; while R51 was Merino 60's.

Group C: N40–N50 and D40–D52 were normal and doggy staples from the same Western District (Victoria) non-Peppin Merino flock.

(ii) *Staining*.—The staples were tied in the middle of the fibres with a cotton thread to prevent felting. They were then cleaned by first degreasing in hot benzene-methanol azeotropic mixture (approx. 2:3 by volume, fractionated, b. p. 59.5°C) in a Soxhlet apparatus for about 12 syphonings. After drying off the solvent, the wool was washed in three 100-ml lots of distilled water at 50°C.

The cleaned wool was then dyed with methylene blue at the boil for 30 min with a liquor: wool ratio of 150 ml to 0.5 g of cleaned wool. The dye-bath consisted

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of Kolthoff buffer at pH 7.4 (82.5 ml of 0.1M KH_2PO_4 mixed with 67.5 ml of 0.05M borax), containing also 0.015% w/v methylene blue. The wool was "washed-off" briefly in distilled water.

The dyeing conditions were arrived at after several experiments, and are believed to be an improvement on those of Fraser and Rogers (1955) in that the dyeing, although showing strong differentiation (staining the orthocortex well but not the paracortex), takes place from a weaker solution of the methylene blue and is fast to the washing-off stage.

The methylene blue used was at first a sample kindly supplied by Dr. R. D. B. Fraser, and later one manufactured by I.C.I.A.N.Z. Ltd., grade 2BN150.

TABLE 1
PERCENTAGE OF PARACORTEX IN NORMAL AND "DOGGY" MERINO WOOLS

Sample No.	Mean Percentage Paracortex		Standard Error
	Normal Wools	Doggy Wools	
R49 N	39.3		1.2
R49 D		41.6	1.0
R50 N	40.8		0.8
R50 D		43.8	1.2
R51 N	41.5		0.8
R51 D		40.4	1.1
N40-N50	35.4-43.8, mean 38.4		Mean 0.7
D40-D52		38.3-48.0, mean 43.6	Mean 1.0
Mean of the means	39.3	43.5	

(iii) *Methods of Estimation.*—Cross sections from the dyed wool, selected as of suitable thickness and uniformity, were projected at a magnification of 500 diameters on to millimetre graph paper and the periphery of the fibres and the lines of demarcation between stained and unstained parts were drawn by pencil. The proportion of the asymmetry was estimated in each fibre by counting the squares enclosed and calculating the percentage of paracortex. From 60 to 73 fibres were thus estimated in each sample.

Results and Discussion

The means of the estimates of the percentage of paracortex in the normal and doggy Merino wools are assembled in Table 1. The mean of all the means of the normal wools is 39.3% while that for all the doggy wools is 43.5%. The difference between these overall means, when compared with the standard error of the difference, is found to be statistically highly significant. The overall mean percentage of paracortex in the doggy wools was, however, notably lower than that obtained

by Ahmad and Lang (1957), possibly because of greater admixture of normal fibres in these doggy samples, as was also indicated by the reduction of occurrence of D to H types of segmentation, found by Glynn, Lang, and Wardle (1960).

The author desires to acknowledge the assistance of the Commonwealth Wool Committee for supporting this work by grant, and of Mrs. L. Windmill for laboratory assistance. He especially wishes to thank Dr. W. R. Lang, Textile College, Gordon Institute of Technology, for helpful discussions.

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THE VISUAL PIGMENT OF AN ISOPOD CRUSTACEAN*

By M. H. BRIGGS†

Visual pigments have been isolated from only five crustacean species to date—*Euphausia pacifica* (Kampa 1955), *Homarus americanus* (Wald and Hubbard 1957), *Meganyctiphanes norvegica* (Fisher and Goldie 1959), and *Hemigrapsus edwardsii* and *Leptograpsus variegatus* (Briggs 1961). This paper reports preliminary findings on the visual pigment of *Porcellio scaber*. This appears to be the first extraction of a visual pigment from the Isopoda.

Heads were removed from between 600 and 700 specimens of *P. scaber* collected locally in the Upper Hutt Valley, N.Z., the crustaceans being first adapted to the dark overnight. All the following extraction procedures were conducted with a dim red light as the only source of illumination.

The tissues were ground with a pestle and mortar, and then with an all-glass Potter-Elvehjem homogenizer. The product was extracted repeatedly with petroleum ether to remove free carotenoids. The resulting tissues were extracted with 2% digitonin and the mixture filtered through sintered glass. The absorption spectrum of a portion of this extract was determined immediately. The solution then was exposed to sunlight for 30 min and the spectrum again determined. The spectrum of another portion of the original extract that had been stored in darkness for 30 min also was determined. No changes in the spectrum of this solution were detected, but the spectrum of the solution which had been exposed to sunlight had changed considerably. A difference spectrum was obtained by subtracting optical density readings of the bleached solution from those of the original extract. This spectrum is given in Figure 1. Maximum absorption is at *c.* 480 mμ.

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Extracts of bleached and unbleached solutions were made with acetone, and also with ethyl ether. These extracts were evaporated and the residues taken up in chloroform. The absorption spectra of these solutions were determined before and after a Carr-Price reaction. Both acetone extracts contained vitamin A₁ and retinene₁ (absorption maxima at 325 and 366 m μ before the Carr-Price reaction, and at 618 and 666 m μ afterwards). Vitamin A₁ was present in the ether extracts of both bleached and unbleached solutions, but retinene₁ was detected only in the ether extract of the bleached solution.

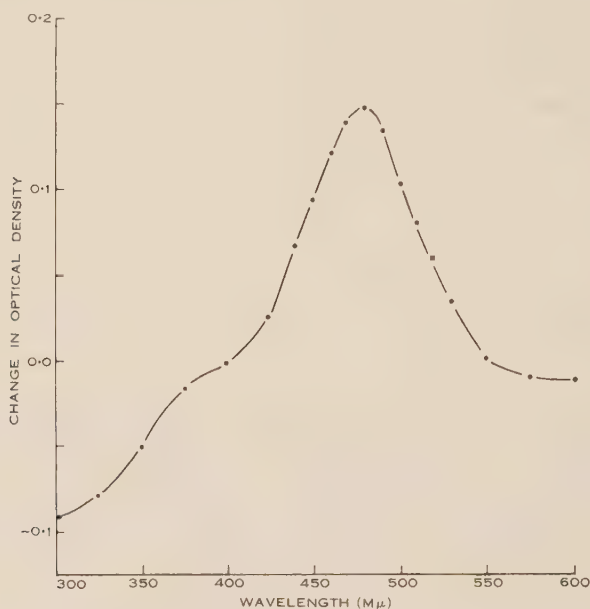


Fig. 1.—Difference spectrum of *Porcellio scaber* rhodopsin.

These results indicate that a visual pigment of *P. scaber* is a compound extractable with digitonin solution, having a $\lambda_{\text{max.}}$ at *c.* 480 m μ , and releasing retinene₁ on exposure to light. It is likely that the compound is a rhodopsin of the now familiar type (Wald 1960).

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THE COUPLING OF CELLOBIASE AND PEROXIDASE BY GLUCOSE OXIDASE*

By M. A. JERMYN†

Youatt (1958) has described a method for estimating cellobiase activity that uses the coupled glucose oxidase–catalase system to determine manometrically the amount of glucose liberated from cellobiose by the enzyme. The high specificity of glucose oxidase makes it the reagent of choice for the otherwise difficult determination of glucose in the presence of excess cellobiose. The hydrogen peroxide produced by glucose oxidase can, however, also be demonstrated qualitatively by the use of peroxidase and both Eyer, Linzenmeier, and von Schrader (1957) and Huggett and Nixon (1957) have described sensitive quantitative glucose oxidase–peroxidase systems for determining glucose as such.

The most easily available source of a powerful peroxidase was the serum from *Ficus macrophylla* latex (Jermyn and Thomas 1954) which has accordingly been used, suitably diluted, throughout this work.

Rationale

Youatt (1958) has shown pH 5.4 to be the optimal pH for the activity of a system containing glucose oxidase and the cellobiase of *Stachybotrys atra*. The reaction between peroxidase and substrate at this pH must meet the following conditions:

- (1) It must be sensitive to very small concentrations of H_2O_2 since the peroxide formed in the system is the result of two sequential reactions, the rate of one of which (cellobiose hydrolysis) cannot be increased at will.
- (2) It must give optical densities at least approximately proportional to H_2O_2 concentration.
- (3) It must give a stable colour.

About 100 potential substrates were tested. Condition (1) eliminated all but four substances as not giving substantial reactions with 10^{-4}M H_2O_2 in pH 5.4 McIlvaine buffer at 28°C. These were *o*- and *p*-phenylenediamine, *o*-aminophenol, and *p*-diethylaminoaniline. Condition (2) eliminated the last of these.

The products from all three had their highest adsorption peaks in acid solution but the optical density of the *o*-aminophenol solutions continued to change rapidly under these conditions, both in the presence and absence of enzyme. This left the phenylenediamines as possible substrates of which the *o*-isomer was chosen as giving the highest adsorption maximum and an oxidation product (phenazine) of known structure and stability. The final acidification used to attain the maximum optical density also had the effect of stopping all enzymic actions.

Hydrogen peroxide (10^{-5} – 10^{-4}M H_2O_2) reacted completely within a minute or two with *o*-phenylenediamine at the concentration finally used, *c.* 0.02%, in the

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presence of peroxidase. When the reaction was allowed to proceed for 5 min, and the mixture then adjusted below pH 1 with HCl, the final optical densities showed a linear dependence on H_2O_2 concentration and the method would be practicable for estimation of traces of peroxide.

The concentration of glucose oxidase to be used was fixed by the arbitrary criterion that, when coupled to peroxidase and *o*-phenylenediamine in the presence of 10^{-4}M glucose, the system should develop an optical density of about unity within 10 min, i.e. the glucose was almost completely oxidized in this time. This amount of glucose, in its turn, can be produced from cellobiose in a few minutes by cellobiase in concentrations that can be readily attained (Youatt 1958).

When an attempt was made to extend the system thus developed by adding the cellobiose-cellobiase step, two difficulties were encountered. In the absence of added cellobiose, the system of three enzymes was found to cause a rapid development of colour in a solution of *o*-phenylenediamine although no combination of two of them would give this effect. Since all three preparations (snail-gut cellobiase, fungal glucose oxidase, *Ficus* peroxidase) were unpurified, it appeared that some coupling of components was taking place to give an amine oxidase system. Such oxidases usually depend on a heavy metal ion, often copper, for activity, and a variety of complexing agents was therefore tested to see whether they would eliminate the effect. Cysteine was found to be highly effective for this purpose, not only eliminating the amine oxidase effect but also much retarding the slow non-specific discoloration of *o*-phenylenediamine solution. The peroxidase activity was only slightly affected by 0.002% cysteine.

Addition of cellobiose to the system without cellobiase also leads to colour development. This phenomenon occurred at much the same rate for a variety of cellobiose samples and does not appear to be due in any great degree to the random contamination of cellobiose by glucose. Keilin and Hartree (1948) cite the comparative rates of oxidation of D-glucose and cellobiose by "notatin" at 0.05M substrate as 100 to 0.09 but comparative Michaelis constants are not given. At the concentrations (c. 10^{-3}M) needed to give maximum rates with *S. atra* cellobiase, the rate of cellobiose oxidation by glucose oxidase was found to be strongly dependent on substrate concentration. The cellobiose concentration finally adopted in the assay (1.0 mg/ml) was chosen as the best compromise between the need to maximize one process and minimize the other.

Reagents

(i) *Buffer*.—McIlvaine buffer, pH 5.4.

(ii) *Peroxidase*.—The serum produced by high-speed centrifugation of *F. macrophylla* latex is divided into 1-ml lots which are stored at -20°C until required; one such lot is diluted to 50 ml with buffer. The diluted solution (PO) keeps its activity for some days in the refrigerator.

(iii) *Glucose Oxidase*.—600 mg of glucose oxidase (Sigma Chemical Co., U.S.A.) is suspended in 20 ml of buffer and the solution cleared by filtration. The filtrate (GO) can be stored a day or two in the refrigerator.

(iv) *o*-Phenylenediamine Hydrochloride (OPD).—A single recrystallization from concentrated hydrochloric acid after decolorization with charcoal gives a white crystalline product. After washing with the acid and a short drying in the oven, this white hydrochloride can be stored indefinitely in the dark at -20°C without visible deterioration.

(v) *OPD Solution*.—100 mg of *o*-phenylenediamine hydrochloride and 10 mg of commercial L-cysteine hydrochloride are dissolved in 50 ml of buffer. This solution must be prepared afresh daily.

(vi) *Cellobiose Solution*.—400 mg of cellobiose dissolved in 100 ml of buffer.

Method

The following solutions (in ml) are added to the tubes in any convenient order, A and B serving for a series of assays:

A		B		C		D	
General Blank		Cellobiose Blank		Enzyme Blank		Assay	
OPD	0.5	OPD	0.5	OPD	0.5	OPD	0.5
PO	0.5	PO	0.5	PO	0.5	PO	0.5
GO	0.5	GO	0.5	GO	0.5	GO	0.5
Buffer	1.5	Buffer	0.5	Sample	0.5	Sample	0.5
				Buffer	1.0		

The tubes are equilibrated for 10 min at 28°C , and 1 ml of separately equilibrated cellobiose solution is then added to each of tubes B and D. After 40 min further incubation, 2 ml of $0.5N$ HCl is added to all tubes. The optical densities of the contents are read at 471μ against a water blank and $(D-C-B+A)$ measures the enzyme activity. Blank B eliminates the cellobiose oxidase effect, and blank C any residual amine oxidase effect. The colours are moderately stable for some hours, any slow increase in colour in the assay samples being compensated for by a corresponding increase in the blanks.

Measured "enzyme activity" is in general not a linear function of enzyme concentration and a calibration curve is needed to convert optical density into enzyme units. As a result of variations in kinetics this calibration curve will not be identical for cellobiases from different sources. Figure 1 shows the results obtained with cellobiases from snail gut and *S. atra*.

The variation in the kinetics of the system with the source of cellobiase and its non-linearity with varying substrate concentration is inherent in the competition for cellobiose of two enzymes, one fixed (glucose oxidase), and one of varying concentration and affinity (cellobiase). In default of exact knowledge of the properties of the enzymes, the use of the system for enzymic assay has therefore to be calibrated on an empirical basis.

Attempts to assay more dilute enzyme samples by increasing the cellobiose concentration or extending the reaction time lead to much greater rises in the optical density of the "cellobiose blank" than of the assay mixture, e.g. a reaction time of

60 min or a cellobiose concentration of 1% treble the blank but raise the enzyme activity only about 75%. The enzyme activity is thus measured as the difference between two large optical densities and accuracy is much reduced. Higher enzyme concentrations may be assayed by lowering the cellobiose concentration or the reaction time (t), or both, the measured enzyme activity of a given sample being approximately proportional to t^2 and [cellobiose]¹. A new calibration curve must be prepared and correlated with the old one.

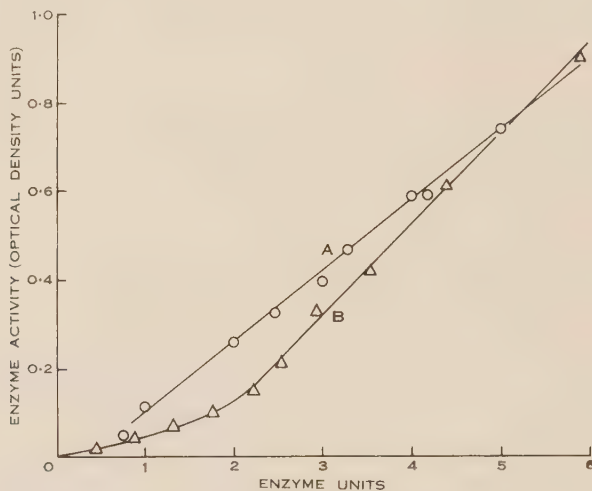


Fig. 1.—Calibration curves for the cellobiases of (A) snail gut and (B) *Stachybotrys atra* under the standard conditions. The values of the "enzyme units" have been adjusted to give approximately equal enzyme activities at 5 enzyme units.

The tediousness of the necessary calibrations gives the present method little or no advantage over the manometric method for the accurate determination of cellobiase activities. It has, however, proved very useful in practice for the rapid assessment of relative activities in large numbers of culture samples with enzyme activities at all levels from very high to very low.

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THE CONTROL OF TILLERING IN THE BARLEY PLANT

I. THE PATTERN OF TILLERING AND ITS RELATION TO NUTRIENT SUPPLY

By D. ASPINALL*

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Summary

The tillering patterns of barley plants were followed from germination until grain maturity under a variety of nutrient regimes. Where nutrients were supplied solely before germination, tiller emergence was restricted to two periods in the development of the plants, and the extent and duration of tillering in the first phase was governed by nutrient supply. The period of non-tillering which followed was not due to the absence of tiller buds and could be terminated at any time by the application of nutrients; the recommencement of tillering late in the development of the plant was not directly related to the development of the early tillers.

When nutrients were renewed throughout growth, tillering was essentially continuous. An early phase of tillering which approached an exponential rate at high nutrient levels was succeeded by a practically constant rate of tillering for the remainder of the experiment.

These relationships are discussed with respect to the control of tillering and the annual habit of growth.

I. INTRODUCTION

Since the pioneer studies of Engledow and Wadham (1923), the importance of tillering capacity as a determinant of cereal yield has been fully recognized. The control mechanisms governing tiller growth have also been frequently studied (Watson 1936; Gregory 1937; Leopold 1949) but no complete picture has emerged. Gregory (1937) stressed the importance of mineral nutrients as a primary factor in tillering and this has been borne out by agricultural experience (Hunter and Hartley 1938). Leopold (1949), on the other hand, provided evidence for the operation of a system of apical dominance in the cereal plant, the inhibiting influence of the apex on tillering being replaceable by an exogenous source of α -naphthaleneacetic acid. Several hypotheses have been advanced to integrate the effects of apical dominance system on the one hand and of the level of mineral nutrition on the other in dicotyledonous plants (van Overbeek 1938; Went 1939; Gregory and Veale 1957). These workers consider that auxin controls the distribution of "nutrients" (both mineral and otherwise) and that axillary bud growth is governed by the resulting nutrient availability. It would be of interest to ascertain whether a similar system operates within the cereal plant but two factors render this difficult to determine. Firstly the stem apices are concealed and hence difficult to manipulate and, secondly, the tillers rapidly produce adventitious roots and become, at least partially, independent of vascular connections with the remainder of the plant. This second factor results in the system under consideration being partially one of competition between a number of individuals.

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Perennial grasses present very similar problems and the relationships between initiation, growth, and senescence of tillers and the development of the tiller apices have been the subject of several reports (Cooper 1951; Lamp 1952; Langer 1956). The basic difference between these plants and cereals, the perennial habit, has been stated (Cooper and Saeed 1949) to be due to differences in the balance between a tendency to rapid floral initiation and subsequent tiller senescence and a tendency to slow vegetative development. Similar studies, involving the tracing of the life histories of individual tillers, have not been carried out with cereals, although Watson (1936) found that only fruitful tillers survived until harvest in the wheat plant. In his experiments, tillering was suppressed late in development despite adequate nutrition. This suggests that a progressive suppression of tillering with age also contributes to the annual habit of the cereal plant.

It seemed appropriate to initiate a study of the mechanisms controlling tillering in barley with a re-examination of the effects of nutrient supply, especially with reference to the influence of different rates and times of application in relation to development. Attention has been given to correlations between tillering and developmental stage to provide comparisons with the known relationships in perennial grasses, and to form the basis for a more complete understanding of the relationship between apical dominance, apical age, and nutrient supply.

II. EXPERIMENTAL METHODS

(a) *Experiments 1 and 2*

Barley plants (*Hordeum distichum* cv. Pirolina (experiment 1), cv. Prior (experiment 2), were grown in 6-in. pots of compost (John Innes) in an open-sided glass-house. Plants in the first experiment were sown in January and grown until July; in the second experiment they were sown in July and harvested repeatedly until December. The tillers on plants in the first experiment were labelled weekly as they emerged from the sheath of the subtending leaf and the ears were later labelled with the date of anthesis; in the second experiment the tillers were counted weekly on one group of plants while sample plants were taken from another group for dissection. The total number of tiller "buds" (tillers less than the length of the subtending leaf sheath) visible to the eye was recorded from these plants. Replication was sevenfold in the first experiment and sixfold in the second, duplicate samples were taken for tiller-bud determinations.

(b) *Experiments 3 and 4*

Barley plants (cv. Pirolina) were grown in 6-in. earthenware pots containing vermiculite, and resting on a larger container, in the same open-sided glass-house as in the previous experiments. Both experiments commenced in March and ran for approximately 20 weeks. The application of mineral nutrients was made in three basic ways:

- (1) Total amount supplied pre-emergence, none subsequently.
- (2) Total amount split into two or more fractions applied at different intervals through plant development.
- (3) Nutrients replaced completely at weekly intervals.

In all cases the volume of solution applied was such that a certain proportion drained through the vermiculite and collected in the lower container. This solution was recycled through the vermiculite daily. Losses from transpiration and evaporation were corrected by making up the solution in the lower container to the initial volume at least once a week, when the solutions in treatment (3) were replaced, and more frequently when transpiration was rapid. The constituents (g/plant) of the nutrient solution used were as follows: NaNO_3 , 1.82; $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 5.13; KNO_3 , 1.58; $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 3.14; MgSO_4 , 1.85; $\text{FeC}_6\text{H}_5\text{O}_7 \cdot 5\text{H}_2\text{O}$, 0.12; H_3BO_3 , 0.014; MnSO_4 , 0.007; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.0004; MoO_3 , 0.0005; in

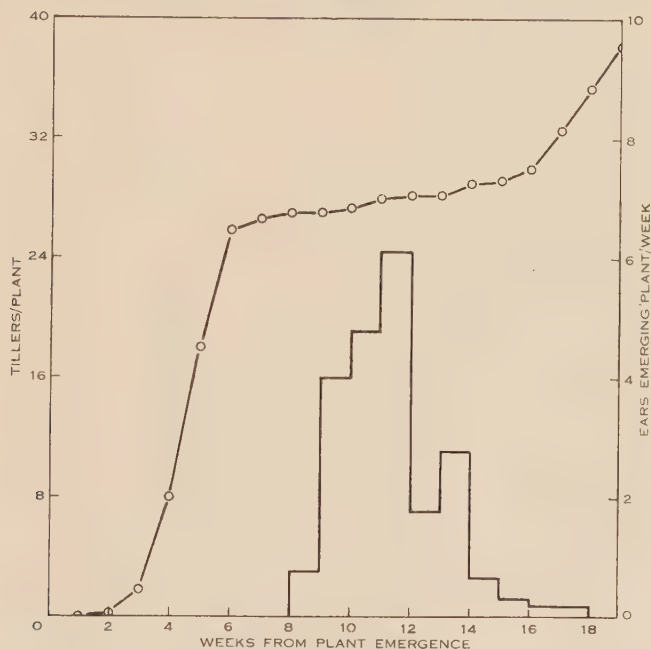


Fig. 1.—Tillering and ear emergence of barley (cv. Pirolina) grown in potting soil, experiment 1. ○ Tiller numbers; histogram: number of ears emerging per plant per week.

500 ml of distilled water.* The total number of tillers and ears were counted weekly and senescent tillers, defined as tillers with all leaves dead, were recorded. Replication was sixfold in the case of experiment 3 and fivefold in experiment 4. Statistical analysis was carried out on the data following a logarithmic transformation and only significant ($P = 0.05$) variations are discussed.

III. EXPERIMENTAL RESULTS

(a) Plants Grown in Soil

The barley plants, cv. Pirolina (experiment 1), showed two clear phases of tiller production (Fig. 1). There was an early rapid rise in the rate of tillering reaching

* Standard solution, referred to subsequently as "100% solution".

a maximum at 5–6 weeks from germination and followed by an equally rapid decline. The plants then entered upon a phase of very slow production of new tillers which lasted for some 9–10 weeks. During this period, the majority of the early tillers came into ear, and finally there was a rapid increase in the rate of tiller production



Fig. 2.—Typical tillers produced on barley (cv. Prior) at the time of ear emergence of the principal tillers.

in the last 3 weeks of grain ripening on the early tillers. The tillers produced in this late stage of growth had extremely reduced laminae on the first leaves (Fig. 2) and in only a few cases did they produce adventitious roots. Frequently they elongated from nodes on the parent tillers which were well above soil level and any roots which did develop were desiccated before reaching the soil. Ear emergence was rapid on these tillers and the ears had fewer spikelets than those on early tillers.

Barley plants (cv. Prior) grown in the second experiment also exhibited a two-phase type of tillering pattern (Fig. 3). Counts of unexpanded tillers and tiller buds were taken from the beginning of the phase of slow tillering. Throughout this phase, until the second increase in tillering rate, the numbers of unexpanded tillers remained relatively constant at between 25 and 35 per plant. The number declined slightly during the second peak of tillering and this decrease was accentuated by the senescence of a number of the tiller buds which did not expand during this period. Of the tiller buds and unexpanded tillers present on the plant at the end of the phase of reduced tillering, approximately one-third elongated in the second phase of tillering and one-third died during this final period. Tiller buds on the newly

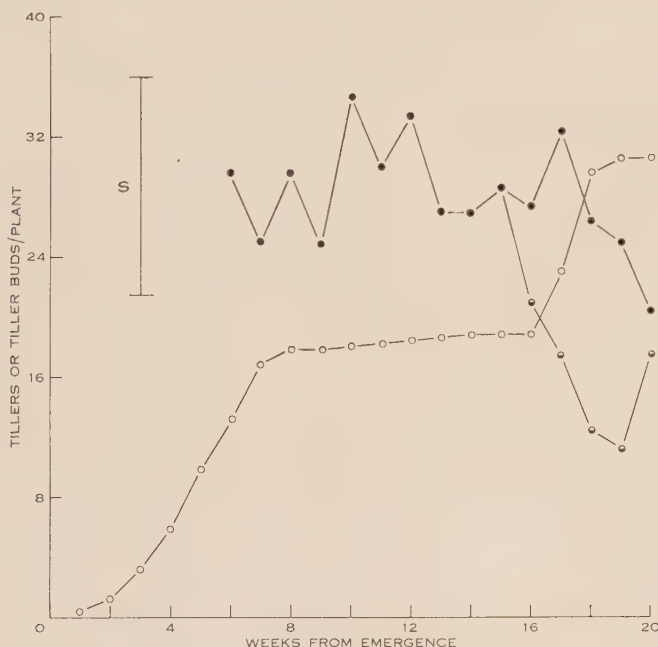


Fig. 3.—Tillers and tiller buds produced by barley (cv. Prior) grown in potting soil, experiment 2. ○ Tiller numbers; ● total number of tiller buds; ● number of living tiller buds. S, significant difference between tiller buds ($P = 0.05$).

emerged tillers had not developed sufficiently to materially contribute to these totals by the end of the experiment. It is clear that tillering during the 9-week period of inactivity was not limited by the number of tiller buds present on the plants.

(b) Plants Grown with Controlled Nutrition

(i) *Experiment 3.*—The nutrient treatments applied to the plants were as follows: (1) All nutrients (100% solution) applied before germination; (2) 50% solution applied initially, 50% solution towards the end of the first phase of tillering (6 weeks postgermination); (3) 50% solution applied initially, 50% solution during the phase of tiller inactivity (10 weeks postgermination); (4) 50% solution applied

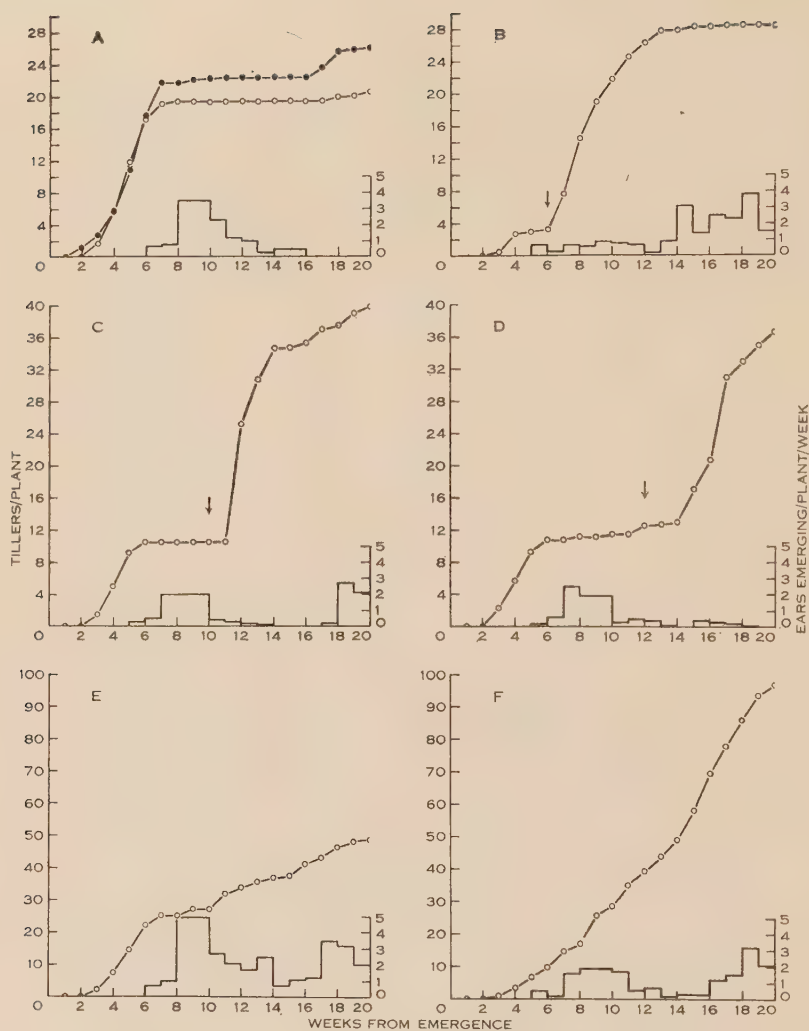


Fig. 4.—Tillering and ear emergence of barley (cv. Pirolina) under varying nutrient regimes, experiment 3. *A*, 100% solution applied before germination. *B*, 50% solution before germination, 50% solution at 6 weeks. *C*, 50% solution before germination, 50% solution at 10 weeks. *D*, 50% solution before germination, 50% solution at 12 weeks. *E*, 5% solution each week. *F*, nutrients replaced completely each week by 100% solution. ○ Tiller numbers; histograms, number of ears emerging per week; ● (*A*), tiller numbers from plants in a previous experiment given a treatment identical to *A*. Arrows indicate the time of application of the second quantity of nutrients in *B*, *C*, and *D*.

initially, 50% solution at the beginning of the second phase of tillering (12 weeks postgermination); (5) 5% solution applied weekly for 20 weeks; (6) nutrients replaced weekly by an amount equivalent to 100% solution.*

Plants supplied with nutrients solely before germination behaved as did the plants grown in soil, giving a two-phase tillering pattern (Fig. 4, *A*). In this experiment, tillering activity in the second phase was slight, amounting to little more than one additional tiller per plant. The tillering curve for an identical treatment in an earlier experiment is given for comparison (Fig. 4, *A*) and here there was greater tillering activity during this second phase. In both cases, this tillering followed upon a prolonged period of absolute inactivity and the tillers which did emerge were similar in appearance to those previously described. Where only half of the total nutrient supply was given initially the first tillering phase tended to cease earlier (Fig. 4, *B, C, D*). Tillering in this early phase was less in treatment (2) than in treatments (3) and (4) although all received the same nutrient supply initially. No explanation can be advanced for this and it did not apparently prevent subsequent tillering responses. The addition of the second amount of nutrients at any of the growth stages had the effect of increasing the tillering rate. The time taken for the plants to respond, however, increased with the age of the plant at the time the application was made. Six weeks after germination there was a response within a week, at 10 weeks this had increased to 2 weeks, and at 12 weeks there was a period of 3 weeks before there was any response. The plants with the least number of tillers at the end of the experiment were those to which all nutrients were applied pre-germination. Approximately the same total number of tillers were produced by plants given two applications whenever the second application was made.

The plants given 5% of the total nutrient supply weekly (treatment (5), Fig. 4, *E*) produced many more tillers than plants given nutrients less frequently (although amounting to an identical total supply). These plants did not pass through any prolonged period of low tillering activity. Where the nutrient supply was maintained at a high level throughout (treatment (6), Fig. 4, *F*) the rate of tillering increased gradually with time. Initially the rate was lower than that of the plants in other treatments (excepting treatment (2)), presumably due to the supra-optimal concentration used for the first few weeks. There was little evidence for any of the tillering phases noted in other treatments or for an eventual cessation of tillering.

Tiller senescence was also influenced by the nutrient supply (Table 1). The criterion of senescence used was a conservative one as it was frequently noted that the upper leaves, and presumably the apical meristem, of a tiller senesced some time before the more mature lower leaves. No dead tillers were recorded on any plants prior to week 11 but thereafter there was a steady loss of tillers from plants supplied with nutrients in two or fewer applications. Few tillers died on plants supplied with nutrients throughout their development, whereas approximately one-third of the tillers were dead by the end of the experiment on plants supplied with nutrients before germination only.

* It was found that this nutrient concentration caused slight wilting when applied; consequently, from the third week onwards it was diluted with a further 500 ml of distilled water per plant.

The time at which the first ear emerged on the plants was approximately the same (6-7 weeks after germination) in all treatments but the subsequent rate of ear emergence was considerably modified by the previous tillering history of the plant. In treatment (1), the majority of the ears emerged between 8 and 11 weeks after germination, during the phase of tiller inactivity. Where two applications of nutrients were made, ear emergence approached a two-phase pattern following the major tillering flushes (Fig. 4, *B, C*). In treatment (4), the experiment was terminated before the tillers induced to emerge by the second application of nutrients had come into ear (Fig. 4, *D*). Continuous applications of a low level of nutrient supply also resulted in a two-phase type of ear-emergence pattern, following closely upon the two early phases of tillering (Fig. 4, *E*). Continuous application of a high

TABLE 1
RELATIONSHIP BETWEEN NUMBERS OF EAR-BEARING TILLERS, THE NUMBERS OF DEAD TILLERS, AND THE RATIO OF TILLERS FLOWERING IN 13 WEEKS OR LESS AT THE TERMINATION OF EXPERIMENT 3

Treatment	Number of Ears Present on Plants, Week 21	Dead Tillers, Week 21	Ratio of Ear Number, Week 21, to Tiller Number, Week 8
1	14.3	6.0	1.0
2	22.2	2.7	1.3
3	17.0	5.5	2.8
4	9.8	5.0	1.4
5	33.2	1.2	0.8
6	19.3	0.2	0.7

level of nutrient supply gave a very prolonged period of ear emergence (Fig. 4, *F*) and it would appear that the period between tiller emergence and ear emergence was prolonged in this treatment as compared with the other lower nutrient levels (Table 1).

(ii) *Experiment 4*.—This experiment was designed to investigate the effects of different levels of nutrient supply, either applied initially or repeatedly, on the pattern of tillering and of ear emergence. The following treatments were applied: (1) All nutrients (100% solution) added before germination (as for treatment (1), experiment 3); (2) 50% solution applied pregermination, no further application; (3) 10% solution applied pregermination, no further application; (4) nutrients replaced weekly by an amount equivalent to 50% solution; (5) nutrients replaced weekly by an amount equivalent to 5% solution; (6) nutrients replaced weekly by an amount equivalent to 1% solution.

In all three treatments in which all the nutrients were applied before germination, the tillering pattern followed the same two-phase form found in previous experiments (Fig. 5, *A*). Reducing the amount of nutrition had the effect of reducing the number of tillers produced in the first phase, hastening the onset of the non-tillering phase and slightly reducing the number of tillers produced in the second phase. The time of emergence of the ears was not influenced by the amount of

nutrients applied but the length of time between tiller emergence and flowering and, in particular, the relationship between the flowering time and the timing of the second tillering phase was very different to that found in previous experiments. Ear emergence was delayed until some 16 weeks after germination and yet the second tillers appeared on the 15th or 16th week as in previous experiments.

The difference in flowering time is most probably due to climatic differences between the periods in which the experiments were carried out. Although experiments 3 and 4 were both sown in March, the temperature during the experimental periods differed in the two years. In 1959 (expt. 3) the mean monthly maxima and minima ($^{\circ}\text{F}$) for the time of the experiment were: March 78.6, 60.5; April 72.7, 56.2; May 65.8, 50.3; June 68.3, 44.6; and in 1960 (expt. 4) they were: March 79.3, 63.0; April 67.3, 53.5; May 63.0, 45.8; June 57.1, 45.4. In addition the

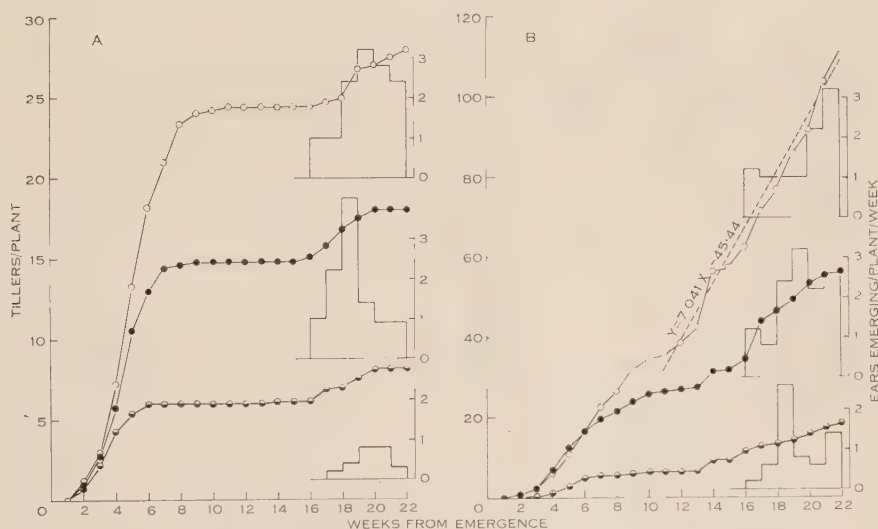


Fig. 5.—Tillering and ear emergence of barley (cv. Pirolina) under varying nutrient regimes, experiment 4. A, all nutrients applied before germination: ○ 100% solution; ● 50% solution; ○ 10% solution; histograms: number of ears emerging per week. B, nutrients replaced weekly: ○ 50% solution; ● 5% solution; ○ 1% solution; histograms: number of ears emerging per week.

incident solar radiation differed considerably between the two years. The difference in the relationship between tillering and flowering, however, is more complex and implies that there can be no direct temporal correlation between ear emergence or stage of ear development and tillering.

Approximately one-third of the tillers produced had died by the end of the experiment in plants given all the nutrients initially (treatment (1) 10 died; (2) 6.2; (3) 3.2), the majority of these tillers reached the final stages of senescence between the 13th and 15th weeks although they had shown signs (e.g. death of upper leaves) of premature senescence at an earlier date.

Where the nutrient supply was replenished regularly, the tillering pattern was determined by the amount supplied (Fig. 5, *B*). At all three levels there was an initial rise in the rate of tillering followed by a fall to a minimal level at approximately 10 weeks postemergence. The tillering rate was not maintained at this minimal level for long, however, and subsequently there was another increase in tillering, being more pronounced at the high levels of nutrition. Ear emergence was not apparently delayed by the repeated application of nutrients, the first ears being recorded on all treatments at 10 weeks postemergence, although the proportion of the early emerging tillers which had flowered by the end of the experiment was lower in the high nutrition series. Tiller senescence was practically negligible in all treatments where nutrients were supplied weekly (treatment (4) 0.4 tillers died; (5) 1.6; (6) 0.6) and the tillers which did senesce did so at a later date (weeks 17–20) than those on plants given nutrients solely before germination.

IV. DISCUSSION

The experiments which have been described in this paper simply provide a background of data against which any general hypothesis on mechanisms underlying tiller control may be judged. It has been confirmed that tillering is very largely controlled by nutrient supply, both with respect to the rate and the pattern of tillering. Thus any scheme which envisages the internal control of tiller-bud elongation by an apical dominance system, and it cannot be denied that such a system does operate in the barley plant (Leopold 1949), must account for the modification of control with changes in nutrient supply.

The pattern of tillering which was established where all nutrients were supplied before germination (e.g. Fig. 1) is similar to those described by Lamp (1952) for *Bromus inermis* and Langer (1956) in *Phleum pratense* and suggests the importance of competition for nutrients within the plant. In the initial phase of tillering, both the rate and number were influenced by nutrient supply (Fig. 5, *A*) and tillering ceased earlier where the nutrient supply was low. As the tillers which were already formed continued to develop, it may be supposed that they were better able to compete for nutrients than were the tiller buds. On the other hand, it is most likely that the death of the apices of a number of the developing tillers, but not of the buds, occurred at the end of the initial phase of tillering and that the senescence of these tiller apices was directly due to a shortage of nutrients as no comparable tiller senescence took place where nutrients were continually supplied. The higher resistance of bud meristems to episodes of nutrient stress is similar to the resistance of young grass seedlings to prolonged periods of nutrient or light stress (Chippindale 1948; Cooper 1948) and may be an inherent property of immature meristems of the Graminaea.

The suppression of tiller-bud development continued for a considerable period but could be relieved at any time by an increase in the nutrient supply (Fig. 4, *B*, *C*, *D*). Wheat plants exhibit a similar period of nutrient-controlled suppression of tillering (Watson 1936) but here, in contrast to the barley plant, the later the stage at which the nutrient supply was increased the fewer was the number of tillers promoted. Watson suggests that competition between the shoots may determine the

pattern, competition becoming more intense with age, but the present data suggests that the state of the inhibited tiller buds following different periods of inactivity should also be taken into account. Increasing the period of inhibition increased the time taken for the buds to respond to an increase in nutrient supply and buds which had withstood a prolonged period of suppression developed into abnormal tillers. These observations parallel data obtained by Mitchell (1953) with *Lolium* spp. and suggests that the difference in response between wheat in Watson's experiments and barley in the present experiments may be due both to the intensity of nutrient stress developed within the plants and to the comparative ability of inhibited tiller buds to withstand such a stress.

The release of tiller-bud suppression during the later stages of growth also indicates the contrast between the situation in barley and in wheat. This release cannot be closely correlated with the development of the early tillers and, hence, it would appear unlikely that the nutrient requirement of the developing ears is the primary factor in its occurrence. Although a decline in nutrient requirement with grain growth could contribute to the effect, some source of nutrients must be tapped to support late tiller growth. The most likely source for the new supply would seem to be the senescing leaves and tillers although there is no direct evidence for this assertion.

No clear phasic pattern of tillering was present in plants supplied with nutrients continuously and the week by week tillering rate was highly irregular, the fluctuations being of a greater magnitude than in the one-application treatments. In no case was an exponential rate of tillering produced, even initially at high nutrient levels, and it cannot be concluded that nutrient availability was even here limiting tillering as (Fig. 5, *B*) a tenfold increase in nutrition did not produce a closer approach to an exponential rate of tillering. A still further increase in nutrient level (Fig. 4, *F*) in a separate experiment caused some inhibition of tillering over this early phase. It is likely that some other factor, for example the number of tiller buds or a progressive change in apical auxin synthesis must initially limit tillering. A reduction in nutrient status to a very low level, however, reduced the tillering rate in this same period, delaying the emergence of even the first tiller (Fig. 5, *B*).

The tillering rate of these plants declined, following the initial rapid phase, at a time which corresponded to the beginning of the non-tillering period in plants grown on nutrients supplied prior to germination. A high nutrient demand by the elongating internodes may account for this decrease as certainly the effect was greater at the lower nutrient levels. Whatever the controlling mechanism, however, its effects were confined to the early stages of internode elongation as tillering recommenced some considerable time before ear emergence. The tillering rate at the highest non-inhibitory nutrient level was constant with time from the 11th week onwards ($y = 7.041x - 45.44$, $P = 0.001$, see Fig. 5, *B*) but at the lower nutrient levels, although there was a general linearity, large individual variations in rate reduced the significance of the trend. The constancy of the rate of tillering at the highest level indicates a state of balance within the plants. If the plants can be considered as a collection of competing meristems, then the activation of a constant number weekly must entail a corresponding decrease in the nutrient requirements

of the older tillers. Another indication of this balance, at all nutrient levels, is the absence of premature tiller senescence indicating that competition between tillers never became sufficiently intense to cause the degeneration of established meristems.

The continued tillering of barley plants, given suitable nutrient regimes, up to and beyond grain maturation on the early tillers raises the question of what determines the annual habit in cereals. Cooper and Saeed (1949) have attributed perennation in *Lolium* spp. to the production of vegetative tillers following the flowering of the early tillers. There is no comparable return to vegetative growth in the barley plant and, indeed, later tillers produce ears more rapidly. On the other hand, the late tillers still produce axillary tiller buds and there is no progressive suppression of tillering with age as noted by Watson (1936). Late tillers which are produced at nodes some distance above ground and consequently produce no adventitious roots will clearly be at a disadvantage and capable of only limited growth but these constitute only a small proportion of the tillers on plants growing under a high constant-renewal regime. It is possible that the annual habit, in a freely tillering variety of cereal, is very largely conditioned by the environment, particularly nutrient supply, and that under certain conditions there may be an approach to perennation. The influence of nutrition on the tillering habit which has been shown to exist in the barley plant would seem to indicate that greater attention should be given to this variable in studies of tillering in perennial grasses. In particular, the great difference between a one-application and a continual-renewal regime may repay investigation in pasture grasses.

V. ACKNOWLEDGMENTS

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STUDIES IN TRANSLOCATION

II. SUBMICROSCOPIC ANATOMY OF THE PHLOEM

By MARGARET DULOY,* F. V. MERCER,* and NELE RATHGEBER*

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Summary

Electron-microscope studies have been made of the cells of the phloem and pericyclic ground parenchyma of the stem of *Cucurbita pepo* (Duchesne). The mature sieve element contains no cytoplasm, either on the side walls or at the sieve plate, such as occurs in other cells. The walls are lined with a parietal layer which encloses an aqueous solution in which are dispersed fibrils of slime. The parietal layer appears to be composed of from one to several membranes, with which are associated numbers of vesicles and a very few mitochondria. The parietal layer lines the sieve plate and extends as a lining to the open sieve pores. There is no closing layer or membrane across the sieve pores. Slime is dispersed more or less uniformly through the lumen, and is continuous through the pores. Dense connecting strands of slime are considered to be an artefact. The individual elements are continuous with each other via the open sieve pores, forming a conduit, the sieve tube. The absence of cytoplasm and organelles suggests that the sieve tube is metabolically inert.

The companion cells, in contrast, are rich in cytoplasm. They are packed with mitochondria and other inclusions, and appear capable of high levels of activity in several aspects of metabolism.

The phloem parenchyma cells are similar in appearance to the pericyclic parenchyma cells and are much less rich in cytoplasmic contents than the companion cells.

I. INTRODUCTION

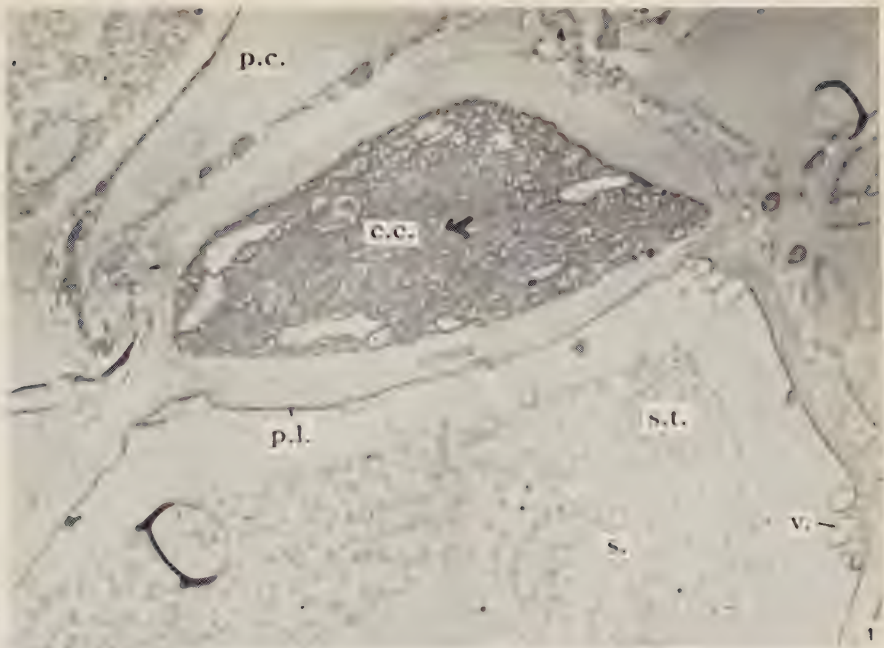
The nature of the angiosperm sieve-element protoplast has been a controversial matter since the time the phloem was first investigated, and currently two divergent viewpoints about its normal state exist. These opposing views are intimately related to the diverging hypotheses of the mechanism of translocation. The "mass flow" hypothesis assumes that the protoplast is "denatured" (i.e. disorganized, non-functional, and freely permeable), and the sieve elements are believed to form a continuous conduit through which a mass flow of translocate occurs. Alternatively, the various "active" hypotheses assume that the protoplast contains functional protoplasm with normal differential permeability, and that the translocate is transported from protoplast to protoplast via connecting strands of cytoplasm.

Most of the recent evidence, discussed in detail later, supports the view that the sieve element contains cytoplasm possibly organized in a complete, though enucleate, protoplast.

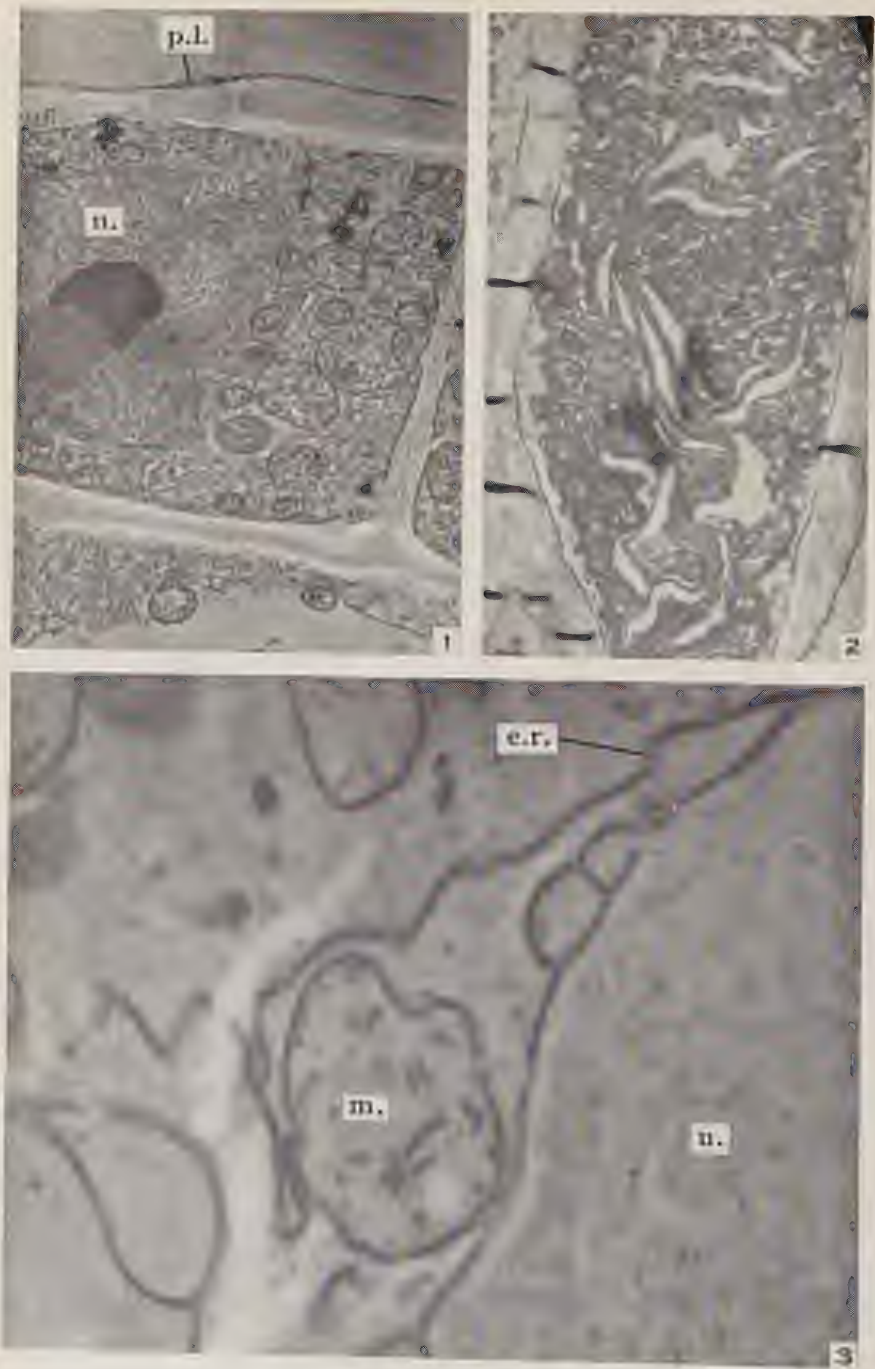
The current studies were undertaken to determine whether the sieve-element protoplast contains such structures as ground cytoplasm, plasmalemma and tonoplast, mitochondria, golgi bodies, and endoplasmic reticulum which are characteristic

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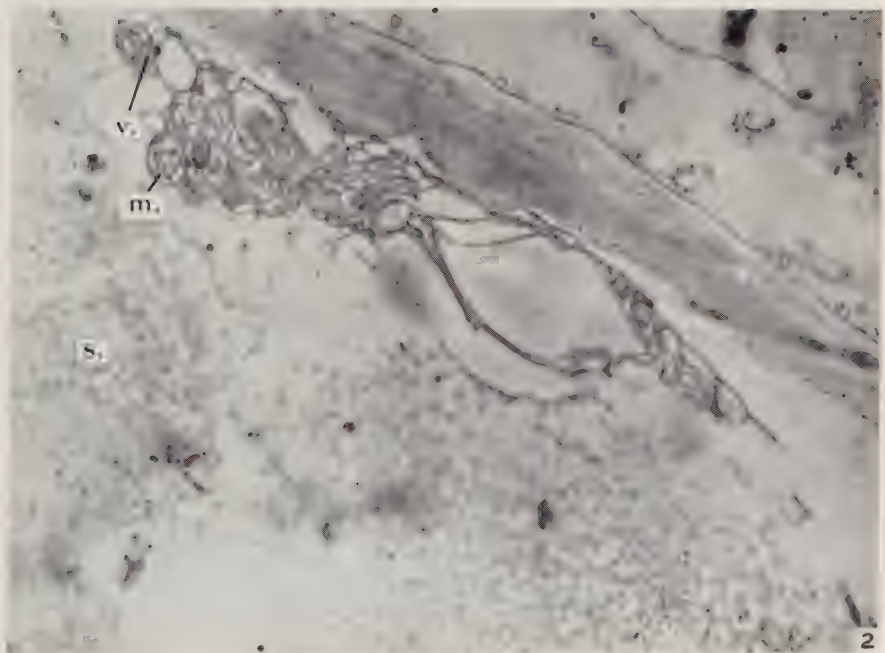
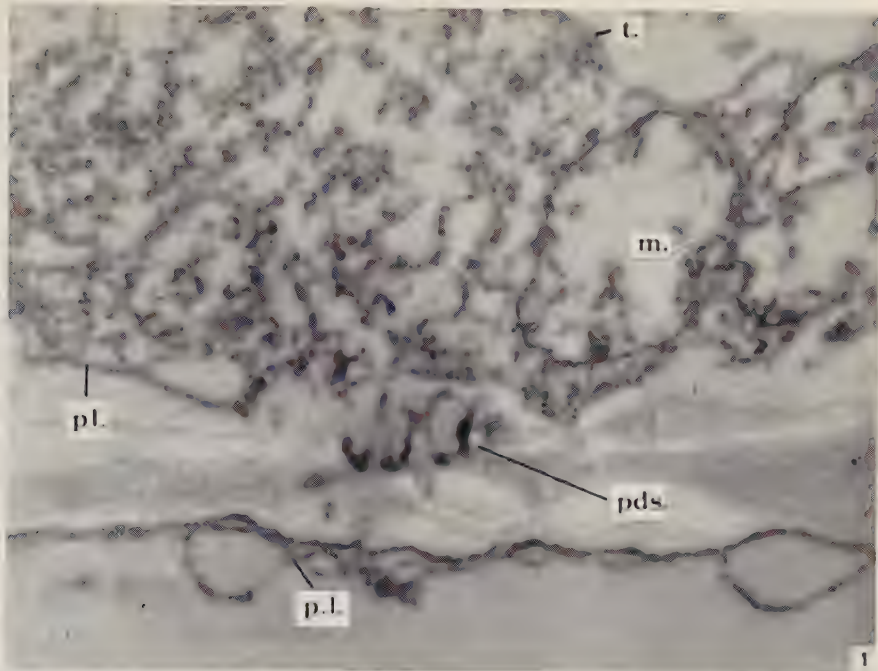
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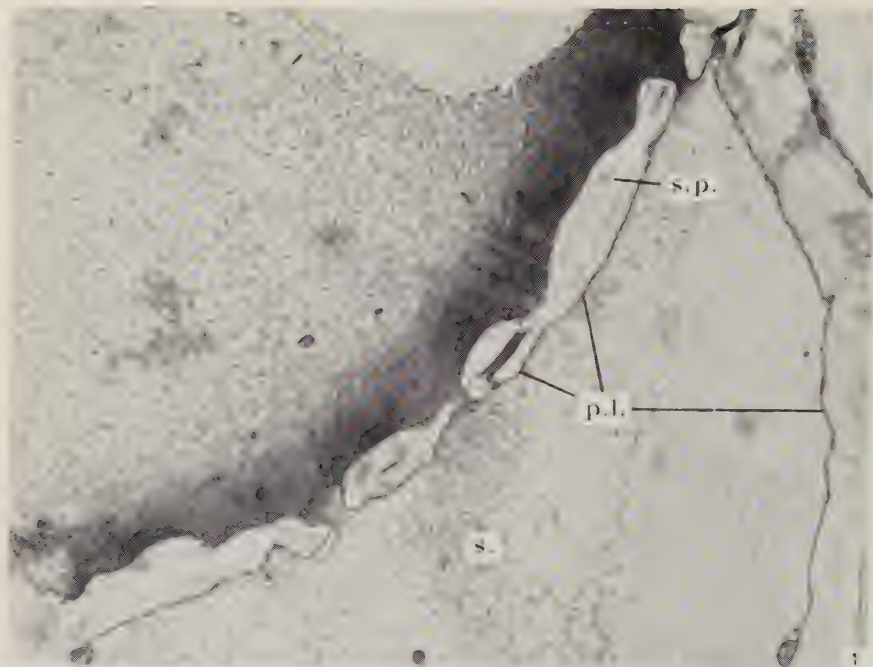
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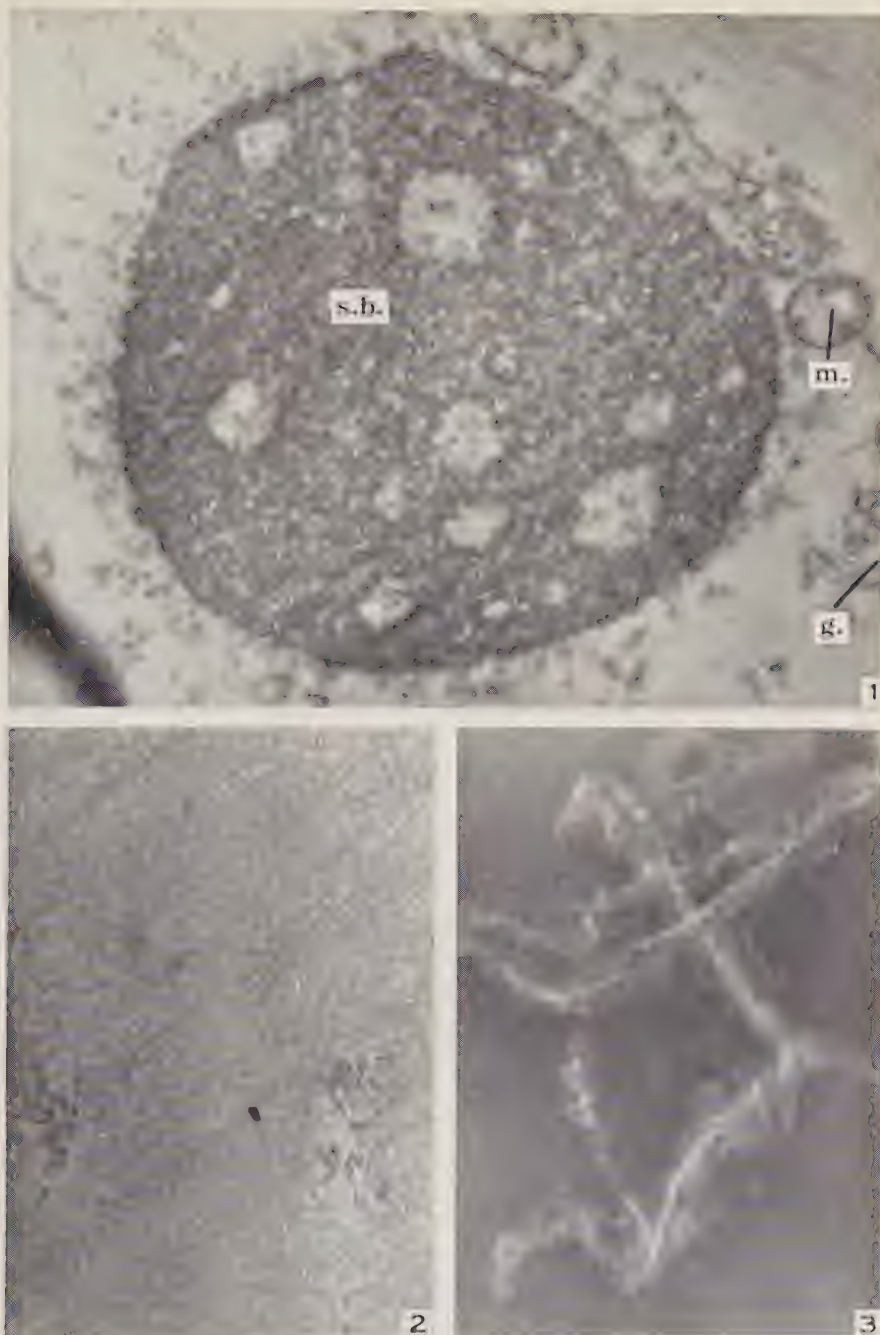
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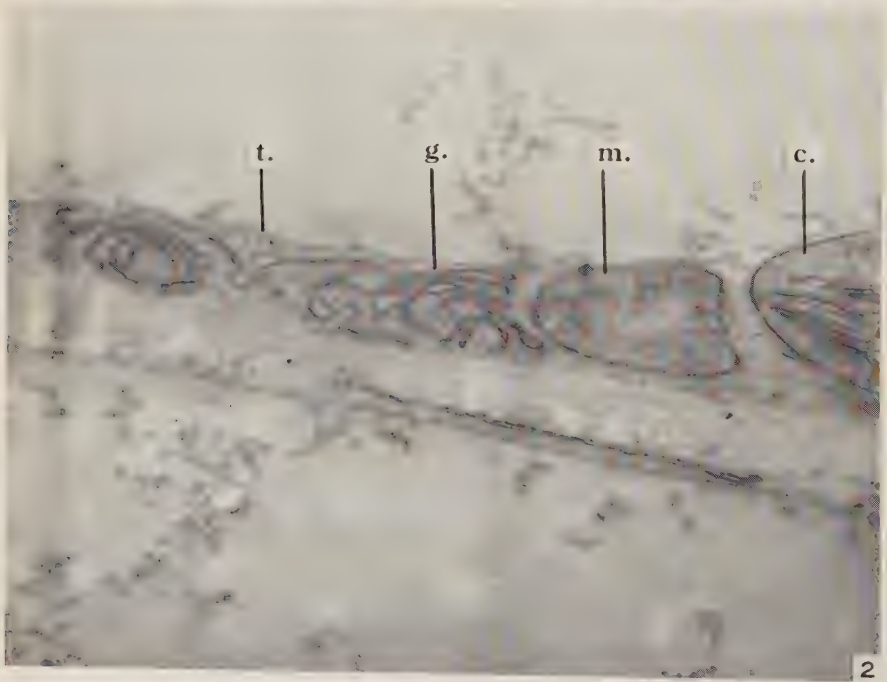
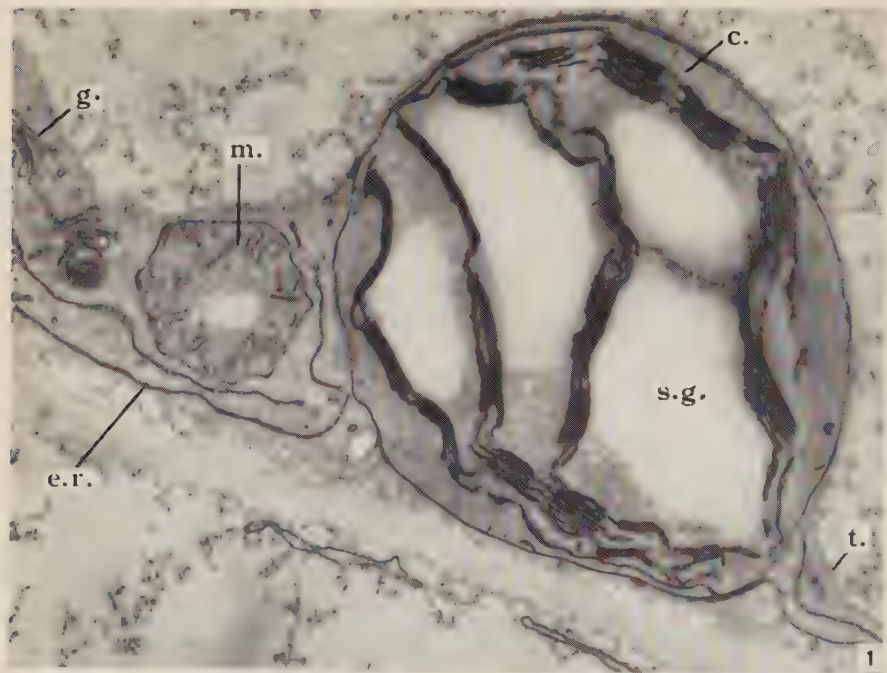
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of the protoplast of other plant cells (Buvat 1958; Whaley, Kephart, and Mollenhauer 1959). The material used was the stem of *Cucurbita pepo* (Duchesne). Electron-microscope studies were made of the sieve element, companion cell, phloem parenchyma, and the pericyclic ground parenchyma. The results lead us to revise the recent views of the structure of the mature sieve-tube element.

II. METHODS

C. pepo plants were grown from seed in open plots containing a mixture of loam, sand, and dried manure. Approximately 15-cm lengths of stem were cut from the plant into buffered 0.2M sucrose solution as used by Currier, Esau, and Cheadle (1955). After 1–2 hr in this solution, 1-cm lengths of stem were cut with a sharp razor-blade from the centres of the 6th, 7th, and 8th expanded internodes from the tip. Vascular bundles of the inner ring, including a small amount of the surrounding parenchyma, were cut individually from the stem pieces while immersed in the solution. Examination with the light-microscope of sections of phloem cut from the material at this stage showed that the sieve-tube elements, except those at the cut ends, were apparently uninjured, and did not show slime plugs. The pieces of vascular bundles were transferred to one of the following fixatives:

- (1) 2% osmic acid in acetate-veronal buffer at pH 7.3, adjusted to a concentration of 0.2M with sucrose. Fixation time was 5 hr.
- (2) 1% potassium permanganate. The fixative was prepared by the method described in "Electron Microscopy" (Anon. 1957) for the osmic acid fixative, the osmic acid being replaced by potassium permanganate. Fixation time was 0.5–1 hr.

After fixation the material was washed several times and dehydrated with a series of ethanol solutions. It was then transferred by several stages to monomethacrylate (1 part methyl to 6 parts butyl methacrylate) and left overnight before embedding in partially polymerized methacrylate. The polymerization was completed in an oven at 40–50°C, or in ultraviolet light. Some of the permanganate-fixed material was embedded in "Araldite" by the method described in "Electron Microscopy". The blocks were trimmed to include only the large-celled region of the phloem. Sections were cut with a Porter-Blum microtome, using a diamond knife, and examined with a Siemens Elmiskop I electron-microscope at 60 or 80 kV.

Droplets of exudate from freshly cut stems were allowed to coagulate and then fixed in 2% osmic acid fixative for 2 hr, and embedded in methacrylate as described for the vascular tissue. Exudate was also examined by the negative staining method of Brenner and Horne (1959). In this case the exudate was collected directly into an approximately equal volume of 4% ammonium acetate solution. The exudate remaining in solution in the buffer was sprayed on to a grid with an equal volume of 2% phosphotungstic acid.

III. RESULTS

The various cell types in the phloem are readily identified under the electron-microscope from a comparison with their appearance under the light-microscope (Plate 1, Fig. 1). The companion cells are small and contain a high proportion of

cytoplasm with numerous cytoplasmic organelles. The phloem parenchyma cells have less cytoplasm and fewer organelles, and frequently contain chloroplasts which do not occur in companion cells, and only very rarely in sieve tubes. Sieve tubes, in contrast, appear almost empty except for slime. The results for each cell type are described in detail below.

(a) *Phloem Parenchyma*

These cells contain large vacuoles so that the cytoplasm forms a narrow layer around most of the periphery of the cell. Larger amounts of ground cytoplasm, however, are associated with the cytoplasmic inclusions, which usually occur in clumps. The cytoplasm is bounded externally by a plasmalemma and internally by a tonoplast. Within the ground cytoplasm the endoplasmic reticulum may be seen as double membranes and small vesicles (Plate 1, Fig. 2; Plate 8, Fig. 1).

The cells contain a number of mitochondria, about $0.5\ \mu$ in width and from 1 to $2\ \mu$ in length. They are bounded by a membrane which in some areas can be seen to be double, and contain a number of cristae in the form of fine twisting tubules. The ground material is somewhat granular in appearance. Bodies are found which are similar to those which have been described as golgi bodies in other plant cells (Buvat 1958; Whaley, Kephart, and Mollenhauer 1959). They consist of bundles of flattened vesicles $0.5\ \mu$ in length and $5\ \mu$ in thickness.

A number of chloroplasts are present, ranging from 1 to $3\ \mu$ in length. They are bounded by a membrane and contain lamellae and grana within a stroma. Small osmiophilic bodies are present, but these are not seen in material fixed in permanganate. Large starch grains are often present.

A nucleus with nucleoli is found in some sections.

Simple pits occur between the phloem parenchyma cells, but pits between phloem parenchyma cells and other cell types have not so far been observed. In the pits numbers of plasmodesmata occur.

(b) *Companion Cells*

These cells (cf. Plates 2, 3, 4, and 5) are rich in cytoplasm, and the vacuole occupies a smaller proportion of the total cell volume than it does in the parenchyma cells. Because the cytoplasmic contents are electron-dense, both the plasmalemma and tonoplast are usually difficult to distinguish. The cytoplasm is packed with organelles, including mitochondria, plastids, and vesicles. The mitochondria are similar to those of the phloem parenchyma cells, but occur in much greater numbers.

The cytoplasm contains a large number of organelles, which vary in size and shape, are larger than mitochondria, contain a granular ground material, and are surrounded by a well-defined membrane. Some of these bodies contain a series of internal double membranes which extend from one part of the boundary membrane to another. Others contain a number of dark and non-staining bodies but only an occasional internal membrane. None of these organelles contains both a series of internal membranes and dark and non-staining bodies as well, suggesting that there may be at least two species of plastid.

A peculiar type of "vacuole" system, different from any yet described in plant cells, is occasionally found (Plate 2, Fig. 2; Plate 3). It consists of elongated vesicles swollen at the tips, somewhat resembling the golgi bodies of other cells. However, these vesicles are extraordinarily long, approximately $12\ \mu$ as compared with $0.5\text{--}1\ \mu$ typical of golgi bodies in plant cells. In addition the vesicles are swollen at intervals along their length and may be partly separated by ground cytoplasm. The ramifications of such a body in the cytoplasm are extensive. In addition to these large vesicle systems many small vesicles and elongated double membranes are found which resemble the endoplasmic reticulum of other cells. It is possible that the large vesicle systems form part of the endoplasmic reticulum which is more elaborate in these cells than in other plant cells.

As the companion cells are small, the nucleus sometimes occupies the major part of the area of the cell in transverse section. The endoplasmic reticulum is seen to be continuous with the outer membrane of the nuclear envelope (Plate 2, Fig. 3). In osmium-fixed material a number of patches of denser material are seen in the nucleus, often at its periphery. These are similar in appearance to what has been interpreted by other workers (E. H. Mercer, unpublished data) to be accumulations of ribonucleic acid (RNA) granules (or nucleoli). The cytoplasm also contains large numbers of granules generally taken to be RNA granules.

(c) *Sieve Element*

On reaching anatomical maturity (defined as the stage at which the slime bodies have broken down and a sieve plate is present) the sieve element (cf. Plates 4–7) contains no cytoplasm, either on the side walls or at the sieve plate, such as is found in other plant cells (Buvat 1958; Whaley, Kephart, and Mollenhauer 1959). The side walls and sieve plate are lined by a parietal membranous layer, enclosing a matrix of slime (Plates 4–6). There appears to be no closing layer or membrane across the pores of the sieve plate. The parietal layer which lines the plate extends also as a peripheral lining to the pores so that it is continuous from one element to the next (Plate 6, Figs. 1, 2, and 3). Where the parietal layer is in contact with dense slime it is frequently difficult to distinguish as a separate layer. Elsewhere, however, it is usually seen as a conspicuous layer which appears to consist of one to several membranes separated by narrow, apparently empty, zones of variable thickness (Plates 4 and 5). The layer as a whole varies in thickness from 200 to about 1000\AA .

A number of vesicles, $0.1\text{--}1.5\ \mu$ in diameter, are found, most of which are associated with the parietal layer while others are scattered, singly or in clumps, throughout the body of the cell.

The mature element contains a very small number of mitochondria, often in clumps interspersed with vesicles. In osmium-fixed material the sieve-element mitochondria differ from those of the companion cells and phloem parenchyma in that the cristae resemble rounded vesicles rather than fine tubules.

On rare occasions chloroplasts are found. These differ from those of the phloem parenchyma in that they lack grana. As is known from studies with the light-microscope (Esau 1950) the mature sieve tube is enucleate.

In the lumen of the mature sieve element is a somewhat granular, fibrous, or reticular substance. From comparative studies of mature and immature elements with light- and electron-microscopes, and from the absence of the inclusions normally found in cytoplasm, we conclude that this material is "slime".

The slime is distributed somewhat unevenly throughout the cell and sometimes, though not always (Plate 6, Fig. 2), tends to be concentrated at the plates. It is quite clear (Plate 6) that the substance filling the sieve pores and hence forming the "connecting strands", is not cytoplasm but is slime. Often the particles of slime appear to be orientated along lines of flow through the sieve pores (Plate 6), i.e. the sieve pores are open, and the slime matrix is continuous from element to element.

The wall of the sieve plate, as seen in transverse section, consists of three zones (Plate 6). These probably correspond with the middle lamella, primary cellulose, and callose layers as identified by light-microscopy.

Numbers of pits, such as described by Frey Wyssling and Müller (1957), occur on the walls between the companion cells and the sieve tubes. Plasmodesmata are present in the pits on the side of the wall towards the companion cell. On the side towards the sieve tube, however, there is a simple pore lined by the parietal layer of the sieve tube. Often pits may be seen close together and probably constitute part of a lateral sieve area.

(d) *Phloem Exudate*

Phloem exudate fixed in osmic acid is identical in appearance with the contents of the sieve tubes (Plate 7, Fig. 2). It contains only a material resembling the slime seen in the sieve elements, and a few vesicles. The vesicles are similar in structure to those of the intact sieve element. The exudate contains no cytoplasmic organelles such as might be expected if the exudate were derived from a normal cytoplasm which is displaced as exudation occurs. The slime appears to consist of fibrillar particles (Plate 7, Fig. 3).

(e) *Immature Elements*

As indicated from studies with the light-microscope, immature sieve elements contain cytoplasm similar to that of other cells (Plate 7, Fig. 1). They possess a ground cytoplasm with an endoplasmic reticulum, a plasmalemma and tonoplast, and contain inclusions such as mitochondria and golgi bodies found in other cell types. In addition the cells contain slime bodies. These have a dense granular structure. Both the number per cell and the size of the slime bodies is very variable, as has been observed with the light-microscope (Crafts 1932). As reported by Crafts, the slime bodies are "vacuolated", that is they contain less dense, or even apparently empty spaces within their matrix.

In the present studies no observations were made of intermediate stages of the breakdown of either slime bodies or cytoplasm. Hence it is not known whether the parietal layer of the mature sieve element is derived from one or more of the cytoplasmic membranes of the immature element, or whether it is formed as a new structure as the element matures.

(f) *Ground Parenchyma*

The cytoplasm of the ground parenchyma (Plate 8, Fig. 2) is identical in appearance to that of the phloem parenchyma which has already been described. It would appear that the main difference between the two types of parenchyma is a difference in shape and volume which can be seen with the light-microscope. Owing to the larger volume of the cells of the ground parenchyma the cytoplasmic layer probably occupies a smaller proportion of the total cell volume in the ground parenchyma than in the phloem parenchyma.

IV. DISCUSSION

Electron-microscope studies have built up a well-documented picture of the structure of the protoplast of meristematic cells, parenchyma cells, and mesophyll cells of several species of angiosperms. In general, the nucleus, mitochondria, golgi bodies, and plastids are distributed in a ground cytoplasm which is bounded externally by the plasmalemma and, in vacuolated cells, internally by the tonoplast. The cytoplasm is permeated by an elaborate membrane system, the endoplasmic reticulum which is apparently continuous with the boundary and nuclear membranes. RNA granules occur scattered through the ground cytoplasm or are attached to the reticulum. Thus the plant cell protoplast is characterized by an elaborate submicroscopic structure (see Mercer 1960).

The observations described in this paper show that the structure of the protoplasts of the pericyclic cells, the phloem parenchyma, companion cells, and immature sieve elements of *C. pepo* are generally similar to those of other living plant cells. In contrast, the structure of the protoplast of the mature sieve element differs from that of other plant cells in several important aspects, including the absence of a ground cytoplasm, the scarcity of organelles, and the presence of slime.

(a) *Ground Cytoplasm*

In the mature element the ground cytoplasm appears to be completely absent.

(b) *Cytoplasmic Membrane Systems*

The only membrane systems in the mature sieve element are those which constitute the parietal layer and vesicles. These are most probably derived from one or more of the membrane systems (plasmalemma, tonoplast, and endoplasmic reticulum) of the immature element. The possibility remains, however, that they are formed as new structures as the element matures. Until their origin is clarified it is probably better to avoid such terms as tonoplast and plasmalemma to describe the structure of the lining layer. New terms may be needed if the layer is not derived directly from the plasmalemma or tonoplast, but until the necessary information is obtained we suggest describing the structure as the parietal layer.

(c) *Organelles*

A small number of mitochondria and very rarely a few chloroplasts occur in the mature sieve tube. These organelles are associated with the parietal layer, being

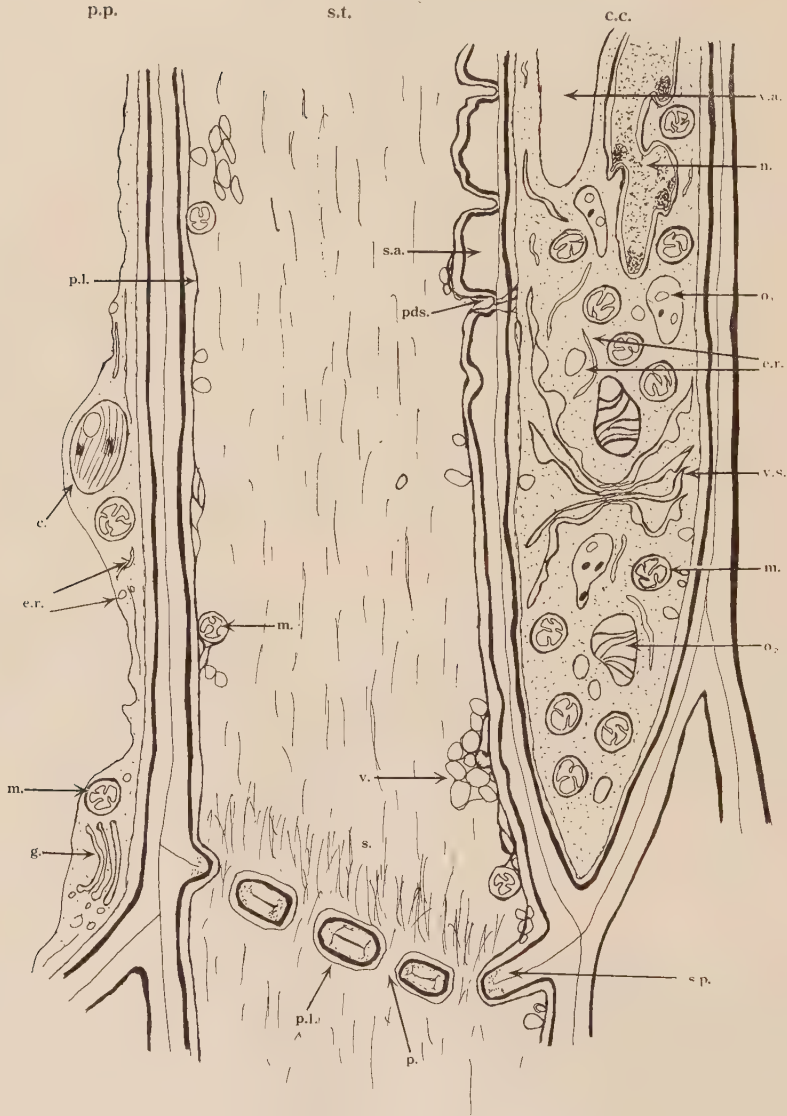


Fig. 1.—Diagram illustrating longitudinal section of phloem with portion of a phloem parenchyma cell (*p.p.*), sieve tube (*s.t.*), and companion cell (*c.c.*). Longitudinal section. Details include chloroplast (*c.*), endoplasmic reticulum (*e.r.*), mitochondria (*m.*), golgi body (*g.*), vacuoles (*va.*), nucleus (*n.*), unidentified organelles, possibly plastids, of companion cells (*o*₁, *o*₂), vacuole system (*v.s.*), parietal layer (*p.l.*) lining wall and sieve pores, drawn away from wall to show continuity of layer, vesicles (*v.*), slime (*s.*), sieve plate (*s.p.*), sieve pore (*p.*), sieve area (*s.a.*), and plasmodesmata (*pds.*).

frequently enmeshed in this membrane system. McGivern (1957) has demonstrated by histochemical means that apparently functional mitochondria occur in clumps in mature sieve tubes, suggesting that the clumping of mitochondria observed by us in the sieve tubes of *C. pepo* is not an artefact of fixation.

(d) *Slime and the Continuity of the Sieve Elements*

The only material in the lumen of the sieve tubes is a granular or reticular substance which by virtue of its appearance and distribution we identify as slime. Frequently in electron-micrographs the slime is seen as a dense layer on the sieve plates and in the sieve pores. Unfortunately the exact distribution *in vivo* is not known. From studies with the light-microscope it is concluded that microscopic accumulations of slime on the plate are artefacts (Esau 1950). In the current studies light-microscope examination of the tissue showed that the contents remained intact during the period for which the tissue was equilibrating with the sucrose solution after cutting from the vine. However, after fixation a displacement of contents, of varying severity, was observed within the sieve elements. Under these conditions it seems likely that a movement of solution between sieve elements would occur, allowing the sieve plates to exert a "filtering" action on the slime, causing it to compact at the plate and in the pores. The lines of flow frequently seen in the slime in the vicinity of the plates is consistent with such an action (Plate 6, Fig. 2). The commonly occurring dense layer of slime at the plates and in the pores, therefore, may be an artefact of fixation. On the evidence so far available we consider it more probable that *in vivo* the slime is distributed more or less uniformly throughout the length of the element (Plate 6, Fig. 2) rather than compacted at the sieve pores, such as shown in Plate 6, Figure 3. In any case it would appear that it is slime rather than cytoplasm which is continuous from one element to the next through the open sieve pores. There is no closing layer or membrane across the sieve pores and the parietal layer which lines the side walls and sieve plate extends also as a lining layer to the pores. Hence both the parietal layer and the slime are continuous from element to element. The individual members of a mature sieve tube are thus much more intimately connected to one another than are most other plant cells. Furthermore the connections are not by strands of cytoplasm as in plasmodesmata, but by slime.

Presumably *in vivo* a solution containing the translocate is associated with the slime. This solution would be continuous from element to element throughout the length of the sieve tube, and since the boundary membranes cannot at the moment be regarded as homologous with a tonoplast, it is doubtful whether the term "vacuole" should be used to describe this solution phase in the sieve tubes.

The structure of the mature sieve element as deduced from the current studies may be summarized as follows: the sieve elements cannot be regarded as discrete entities, but must be thought of as segments of a conduit, the sieve tube, which contains a continuum of solution and slime enclosed by the membranous parietal layer (Fig. 1).

This view of the structure of the mature sieve element is at variance with the conclusion reached from recent investigations, namely that the lining layer and

connecting strands are composed of cytoplasm. This conclusion was based on three lines of evidence.

First, from electron-microscope observations Hepton, Preston, and Ripley (1955), Preston (1958), and Schumacher and Kollman (1959) concluded that the material of the parietal layer and connecting strands was composed of cytoplasm.

Secondly, the demonstration by Currier, Esau, and Cheadle (1955) of the plasmolysability of the sieve element indicated that the sieve-element protoplast is not "denatured" and freely permeable but does possess the property of differential permeability at least along the lateral walls.

Thirdly, the high respiration rates in the phloem calculated by Kursanov and Turkina (1952) and Kursanov, Turkina, and Dubenina (1953) have been taken to indicate the presence of a metabolically active protoplast in the sieve element (Preston 1958; Spanner 1958).

Each of these lines of evidence is discussed below together with the evidence of the current studies.

The identification of substances with the electron-microscope is not always straightforward, and it is doubtful whether a shadowing technique such as was used by Hepton, Preston, and Ripley (1955) would reveal the distinction between slime and cytoplasm. As far as can be assessed from their published data the substance in the sieve elements more closely resembles the substance interpreted by us as slime. It has the granular, fibrillar appearance of slime rather than the complex structure of cytoplasm.

Similarly, the substance identified as cytoplasm by Preston (1958) and Schumacher and Kollman (1959) could be slime. Their electron-micrographs show that the substance is extremely electron-dense. It is not possible to identify the material from the published data, but there are no structural features to suggest that it is cytoplasm. The presence of a small number of organelles in the sieve element does not necessarily imply that a well-organized cytoplasm also exists, particularly when the organelles are swollen and possibly partially broken down, as they are in some of the published electron-micrographs.

As has been pointed out by Currier, Esau, and Cheadle (1955) the demonstration of reversible plasmolysis shows that the mature sieve-element protoplast is differentially permeable along its lateral walls, though not necessarily at the sieve plates. However, there is no reason to doubt that the membranous parietal layer could account for this lateral differential permeability, and that a layer of normal cytoplasm is not essential. The finding of the current studies of the open structure of the sieve pores obviates the problem raised by Currier, Esau, and Cheadle of the apparent anomaly of differential permeability in the sieve tubes and the longitudinal surging flow which can be induced in a series of sieve elements. The continuity of the elements through the pores also accounts for the plasmolysis forms found by Currier, Esau, and Cheadle, namely the sieve-element protoplasts being attached at the sieve plates and concave along the lateral walls.

Finally, from the evidence of the current studies it appears that the high respiratory activity which has been reported for the phloem can be accounted for

largely by the companion cells and phloem parenchyma, containing large numbers of mitochondria, rather than by the sieve elements as was previously suggested (Kursanov, Turkina, and Dubenina 1953). Indeed it seems that for *C. pepo* at least, the high respiration rates that are found in the phloem (Duloy 1960; Duloy and Mercer 1961) cannot be attributed to the sieve tubes, which show a marked scarcity of mitochondria.

The view of the structure of the sieve tube derived from the present study allows explanation of a number of the peculiar properties of the mature sieve element.

The phenomenon of exudation can be explained on the structure proposed here. Assuming a positive turgor exists in the sieve tubes, exudation would occur from the sieve elements following cutting because of the open structure of the pores and the high permeability of the sieve tubes in the longitudinal direction.

The difficulty of detecting the so-called "cytoplasmic layer" in mature elements, with the light-microscope (Currier, Esau and Cheadle 1955), the problem of an ill-defined "vacuole", the paucity of contents in the protoplast as seen under the light-microscope (Esau 1950), and the extreme sensitivity of the sieve-element protoplast to handling, can all be attributed to the extreme thinness of the parietal layer as revealed by electron-microscopy.

The absence of a ground cytoplasm would account for the fact that cytoplasmic streaming has never been observed in mature sieve elements. Again, the lack of ground cytoplasm and the scarcity of organelles might account for the weak tetrazolium reaction reported for mature sieve elements (Bauer 1953) and for the lack of affinity for "plasma" stains (Esau 1950). Similarly, the paucity of enzymes observed by Wanner (1953) in phloem exudate is consistent with the lack of cytoplasm and organelles in the sieve elements.

The absence of a ground cytoplasm may be the result of the absence of a nucleus in the mature element. If in these cells the maintenance of cytoplasmic proteins depends on the continued production of RNA, then it might be expected that the loss of the nucleus could be followed by a loss of the proteins of the ground cytoplasm.

(e) *Comparison of Constituent Cells of the Phloem*

The scarcity of mitochondria and absence of ground cytoplasm indicates that the mature sieve element may be almost completely inert metabolically, and deficient in enzymes.

Again, the phloem parenchyma cells appear to be capable of levels of activity similar to those of the surrounding ground parenchyma, and lower than those likely to be found in the companion cells. The companion cells, in contrast to both sieve tubes and phloem parenchyma, are rich in cytoplasm and its inclusions. They contain large numbers of mitochondria indicating that the cells are capable of high levels of respiratory activity. The companion cells also appear to contain a large amount of RNA material, both in the nucleus and in the cytoplasm. Since the level of protein synthesis in a cell appears closely to parallel the amount of RNA material present (Brachet 1960), the implication is that the companion cells are capable of a high level

of protein synthesis. In addition these cells contain a large number of plastids, the functions of which are at the moment unknown, and an endoplasmic reticulum more elaborate than those found in other plant cells. This cytological evidence strengthens the view that the companion cells, with their close structural relationship with the sieve tubes, may be at least partly responsible for the maintenance of the mature sieve element. Whether or not a high level of metabolism in the companion cells is directly necessary for the process of translocation is still an open question.

(f) *Conclusions*

Three main conclusions can be drawn from the current study about the phloem of *C. pepo*. First, from the distribution of cytoplasm and its inclusions in the cells of the phloem it seems that it is the companion cells rather than the sieve tubes that are responsible for the high levels of metabolic activity that have been found in the phloem. Secondly, there appears to be a reduction of the cytoplasmic contents of the sieve elements to an extremely thin parietal membranous layer, the remainder of the cell being occupied by a solution in which slime is in some way dispersed. Thirdly, the individual mature sieve elements are continuous with one another via the open sieve pores, so forming a conduit, the sieve tube.

V. ACKNOWLEDGMENTS

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EXPLANATION OF PLATES 1–8

PLATE 1

Fig. 1.—Transverse section of phloem showing parenchyma cells (*p.c.*), companion cell (*c.c.*), sieve tube (*s.t.*) with parietal layer (*p.l.*), vesicles (*v.*), and slime (*s.*). $\times 5000$.

Fig. 2.—Portions of two phloem parenchyma cells. They contain a thin peripheral layer of cytoplasm bounded externally by a plasmalemma (*pl.*) and internally by a tonoplast (*t.*). Chloroplasts (*c.*), mitochondria (*m.*), and golgi bodies (*g.*) are seen. $\times 20,000$.

PLATE 2

Fig. 1.—Companion cell with part of neighbouring sieve element above it. The cytoplasm of the companion cell contains numerous mitochondria and plastids, and a few vesicles. The nucleus (*n.*), which occupies a large part of the area of the cell in this section, contains a nucleolus and several other areas of density greater than that of the nucleoplasm (possibly nucleolar material). The sieve element is bounded by a thin parietal layer (*p.l.*) in parts of which two membranes can be seen. $\times 10,000$.

Fig. 2.—Companion cell, showing extensive system of cytoplasmic membranes and vacuoles. $\times 10,000$.

Fig. 3.—Companion cell, showing nucleus (*n.*) at right, bounded by double membrane the outer layer of which is continuous with the endoplasmic reticulum (*e.r.*). The mitochondrion (*m.*) has fine tubular cristae, and an external double membrane which also appears to be continuous with the endoplasmic reticulum. Potassium permanganate-methacrylate. $\times 40,000$.

PLATE 3

Section of companion cell. The cytoplasmic membranes which ramify through the cytoplasm are closely packed, have a parallel arrangement, and enclose an extensive system of vacuoles. $\times c. 20,000$.

PLATE 4

Longitudinal section of sieve element and companion cell. The parietal layer (*p.l.*) of the sieve element is associated with numerous vesicles (*v.*), but no mitochondria are present in this section. Fibrous slime (*s.*) is dispersed throughout the lumen of the element. In contrast to the sieve element, the companion cell contains numerous mitochondria (*m.*) and plastids (*pt.*), and a nucleus (*n.*) with several dense areas (*d.a.*) taken to be nucleolar material. The wall between the sieve element and companion cell shows part of a sieve area, the side towards the sieve element being perforated by pores, while that towards the companion cell shows several plasmodesmata (*pds.*). $\times 15,000$.

PLATE 5

Fig. 1.—Sieve element and companion cell. The companion cell at top shows granules in the cytoplasm, a plasmalemma (*pl.*), and tonoplast (*t.*), and mitochondrion (*m.*). In the sieve element the parietal layer (*p.l.*) can be seen to consist of from one to several membranes, separated at intervals to form vesicles. Part of a sieve area can be seen in the wall between the two cells, with plasmodesmata (*pds.*) on the side towards the companion cell. $\times 20,000$.

Fig. 2.—Sieve element. A number of mitochondria (*m.*), small vesicles (*v.*), and membranes can be seen. This is the largest accumulation of cytoplasmic elements that was found in a mature sieve tube. Slime (*s.*) is dispersed in the lumen of the cell. $\times 10,000$.

PLATE 6

Figs. 1-3.—Longitudinal sections of mature sieve elements in area of sieve plates. The parietal layer (*p.l.*) lines the side walls and sieve plate (*s.p.*) and in some places can be seen to extend as a lining to the sieve pores so that it is continuous from element to element. The parietal layer in the sieve pores is sometimes impossible to distinguish because of the deposits of slime. Slime (*s.*) is dispersed throughout most of the lumen of the element, and is continuous through the sieve pores. The slime sometimes, though not always (Fig. 2) tends to accumulate on one side of the plate. $\times 10,000$.

PLATE 7

Fig. 1.—Immature sieve element, showing slime body (*s.b.*) with small "vacuoles". Mitochondria (*m.*) and golgi bodies (*g.*) can be seen in the cytoplasm. $\times 20,000$.

Fig. 2.—Exudate fixed in osmic acid. $\times 20,000$.

Fig. 3.—Exudate prepared by negative staining with phosphotungstic acid. $\times 80,000$.

PLATE 8

Fig. 1.—Phloem parenchyma cell. A golgi body (*g.*), mitochondrion (*m.*), and chloroplast (*c.*) with starch grains (*s.g.*) can be seen in the cytoplasm, together with elongated membranes of the endoplasmic reticulum (*e.r.*). Part of the tonoplast (*t.*) can be seen towards the right-hand corner of the section (compare with Plate 1, Fig. 2). Potassium permanganate—"Araldite". $\times 20,000$.

Fig. 2.—Ground parenchyma cells. A greater part of the cell has the appearance shown in the lower cell of this figure, where elongated membranes of the endoplasmic reticulum can be seen. At intervals, however, a clump of cytoplasm occurs such as shown in the upper cell, with golgi bodies (*g.*), mitochondria (*m.*), and chloroplasts (*c.*). The cytoplasm is bounded internally by a tonoplast (*t.*). Potassium permanganate—"Araldite". $\times 40,000$.

EFFECTS OF SEVERAL OSMOTIC SUBSTRATES ON THE WATER RELATIONSHIPS OF TOMATO

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Summary

Osmotic substrates, comprising 5- and 10-atm concentrations of potassium nitrate, sodium chloride, mannitol, and sucrose were added to standard culture solutions in order to determine the effect on relative turgidity, DPD, osmotic potential, transpiration, and growth of tomato plants. ^{14}C -labelled mannitol and ^{36}Cl -labelled sodium chloride were incorporated into the high-concentration treatments to provide additional information on solute absorption.

After an initial loss of water content (fresh weight — dry weight) and associated wilting, recovery took place rapidly in all except the mannitol treatments and after 28 hr did not differ significantly from the control. At this time the first stage of the experiment was concluded and all plants were replaced in standard culture solutions.

Recovery of water content and turgor during the first stage was associated with a rapid increase in internal osmotic potential. This proceeded to such an extent that, by the end of stage 1, the internal potential in each treatment exceeded the substrate potential by approximately the same amount as the potential of the control exceeded that of the base nutrient solution. The increase in internal osmotic potential was achieved mainly by solute absorption except in the mannitol treatments in which dehydration appeared to account for almost half of the increase.

Absorption of ^{36}Cl from labelled sodium chloride was closely related to the increase in osmotic potential in the high-concentration sodium chloride treatment, the relative concentration of the isotope in the plant at the end of stage 1 compared with concentration in the substrate being approximately 1 : 1. In the mannitol treatment the relative concentration of ^{14}C was only 1 : 4, confirming that mannitol absorption alone could only account for a small proportion of the total increase.

Following the removal of the osmotic substrates an initially rapid and progressive decline in internal osmotic potential occurred, together with an associated reduction in ^{36}Cl and ^{14}C concentration in the tops of the plant. In both cases this decline could be satisfactorily accounted for by increased water content of the plants, leakage of solutes to the substrate being negligible.

The effect of the osmotic treatments on transpiration appeared to be closely related to changes in volume (expressed as water content), showing an initial severe reduction and subsequent recovery to values close to the control in all except the high-concentration mannitol treatment. Because of the short term nature of the experiment no significant changes in growth (measured as increase in dry weight) could be detected in stage 1. Following the removal of the osmotic substrates however, dry weight increase in the low-concentration sucrose and potassium nitrate treatments was more rapid than in the control and in the high-concentration mannitol treatment was significantly less.

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I. INTRODUCTION

There is now general agreement that the availability of soil water for plant growth decreases progressively as soil moisture stress increases (Richards and Wadleigh 1952) and that the first evidence of decreased growth occurs at quite low stress levels. Evidence to the contrary (Veihmeyer and Hendrickson 1950) is based mostly on field experimentation, the proper interpretation of which is rendered difficult because of the variation which exists between different experiments with respect to root distribution, hydraulic conductivity of the soil water, total depth of the root zone, and other experimental factors.

It has also been proposed by research workers at Riverside, California (Wadleigh and Ayers 1945; Wadleigh 1946) that the total water potential (total soil moisture stress) equals the sum of the soil moisture tension and the osmotic potential of the soil solution. It is normally assumed that this viewpoint requires that the plant acts as an ideal osmometer and that the solutes contributing to the osmotic potential are non-diffusible. It has been widely accepted (Magistad 1945; Bernstein and Hayward 1958), and is embodied in the concept of "physiological dryness" frequently applied to saline soils. It is supported by a considerable volume of experimental evidence indicating reduced water absorption, growth, and metabolism as the substrate concentration increases (Richards and Wadleigh 1952; Kramer 1956). However, it has been challenged by Walter (1955) on the basis that, if the solutes in the substrate or soil solution are freely diffusible, the osmotic potential of the soil solution is balanced by the intake of solutes. Supporting evidence is also available for this view (Eaton 1927, 1942) and, in many respects, it is to be expected from known facts concerning salt absorption (Gauch 1957; Robertson 1958).

More recently Philip (1958) and Bonner (1959) have suggested that the opposing viewpoints could be reconciled by envisaging, in soil-plant water systems, the development of a vapour gap and disruption of liquid phase continuity, at the soil-root interface. It is suggested that prior to the development of such a vapour gap the Walter view should hold, and that the effective soil moisture stress would not include an osmotic component, but subsequently the view of the Riverside workers would be valid and that the effective soil moisture stress would include an osmotic component. The Riverside workers have rejected this view (Bernstein, Gardner, and Richards 1959) on the basis that the observed effects are apparent in culture solution experiments in which no vapour gap can be envisaged and that rates of vapour transport are inadequate to supply the amounts of water required.

If the osmotic potential of the soil solution is a component of the total soil water potential, it must result in the development of a water potential in the plant, of equal magnitude to the sum of soil moisture tension and osmotic potential. Consequently, the measurement of the internal water relations of plants grown on osmotic substrates should provide a ready solution to the controversy. Since no data of this type have been discovered in the literature, a study involving these measurements was conducted with plants grown in culture solutions to which were added osmotically active organic and inorganic substrates to ascertain the nature of the plant response.

Sucrose and mannitol were selected as the organic substrates on the assumption that sucrose is absorbed by plant roots and is readily metabolized in the plant, whereas evidence suggests that mannitol is only slowly absorbed (van Overbeek 1942; Groenewegen and Mills 1960) and is not metabolized to any degree. Potassium nitrate and sodium chloride were selected as the inorganic substrates. Both are readily diffusible but it was thought that the known toxic effects of excess sodium chloride could result in a different order of response.

II. EXPERIMENTAL DETAILS

(a) *Preparation of Material*

Tomato seedlings (cv. Grosse Lisse) sown in sand culture were transferred to standard water culture solutions after 3 weeks and grown to the five-leaf stage, when the average fresh weight of each plant was of the order of 25 g. The culture solution was prepared according to solution 1 of Hoagland and Arnon (1938).

The osmotic substrates were prepared with A.R. reagents to provide osmotic potentials of approximately 5 and 10 atm in the low-concentration and high-concentration treatments respectively. The concentrations appropriate to each solution were computed from data on freezing-point depressions and osmotic coefficients tabulated by Robinson and Stokes (1955) and Hodgman (1955). Because particular interest was associated with the sodium chloride and mannitol treatments, the former was labelled by the addition of 0.1 mc of ^{36}Cl as NaCl and the latter by the addition of 0.1 mc of ^{14}C -labelled D-mannitol with the isotopes at the 1 and 6 positions.

At the commencement of the experimental period, plants selected for uniformity were arranged into nine groups to provide a control in addition to low- and high-concentration treatments of KNO_3 (K_1 , K_2), NaCl (N_1 , N_2), mannitol (M_1 , M_2), and sucrose (S_1 , S_2). Each treatment group consisted of four replications each of 10 plants, and each set of 10 plants was arranged in standard 3 l. containers.

Imposition of the osmotic substrates involved the replacement of the standard culture solutions by the specific substrate treatments. This was achieved in minimum time by having pairs of standard and treatment containers and rapidly transferring each group of 10 plants. Removal of the osmotic substrates involved the reverse procedure.

The experimental period commenced at 10.00 a.m. on June 27, 1960, with the transfer of all plants to the substrate treatments. The plants were exposed to these substrates until 2.00 p.m. on June 28 and then returned to standard culture solutions. The experiment was then continued until 10.00 a.m. on July 1 so that the short-term effects of the exposure to osmotic substrates could be observed. Nine sampling occasions were spread through the experimental period.

(b) *Measurement Techniques*

(i) *Relative Turgidity*.—Relative turgidity was measured using the general technique of Weatherley (1950, 1951) except that (1) leaf disks of 0.8 cm diameter

were employed; (2) the period of floating was 4 hr at constant temperature; (3) during floating the disks were continuously illuminated by a 20-W fluorescent light mounted about 20 cm above the bench top.

(ii) *Water Potential*.—The water potential of the leaf tissue was measured as diffusion pressure deficit (DPD) using the vapour equilibration technique described by Slatyer (1958). The length of time allowed for each determination was 8 hr.

(iii) *Osmotic Potential*.—Measurements were made using the expressed sap method. Although sap expression may introduce some errors, it was thought (Crafts, Currier, and Stocking 1949) that these would be minor compared with those which could arise in the plasmolytic method. The osmotic potential of the leaf tissue sap was determined by (1) snap-freezing the fresh tissue to -20°C ; (2) expressing the sap in a hydraulic press at 140 kg/cm^2 (2000 lb/in^2); (3) measuring freezing-point depression with standard cryoscopic equipment; (4) estimating equivalent osmotic potential using the procedure described by Crafts, Currier, and Stocking (*loc. cit.*).

(iv) *Transpiration and Water Absorption*.—Transpiration was measured by weighing each container at each sampling occasion, and water absorption calculated by adjusting this value by the loss of water represented by the changing fresh weight of the plants. The containers were topped up with water at each sampling occasion.

(v) *Plant Weight*.—On each sampling occasion one plant was moved from each replication of each treatment and the tops and roots immediately placed inside polythene bags, weighed, and transferred to a constant-temperature room. The plants were then partitioned into laminae, petioles, stems, and roots. Although portions of each plant were utilized on the measurements described above, a record of all dry weights was maintained and fresh weight and dry weight for all treatments obtained.

(vi) *Water Content*.—It was desirable to measure changes in water volume so that the effect of solvent volume on osmotic potential and turgor pressure could be estimated. These data were obtained as fresh weight—dry weight from the determination described above and, in the text, use of the word “volume” implies water content determined in this manner.

(vii) *Isotope Concentration*.—On each sampling occasion the plants sampled from the treatments containing ^{14}C and ^{36}Cl were partitioned into leaves (laminae only) and stems (including petioles) and snap-frozen to -20°C . Also on each sampling occasion 10-ml aliquots were pipetted from the substrate solutions. The mannitol and chloride was removed from the thawed plant material by aqueous extraction, involving repeated boiling and filtration until a negligible amount of radioactive material remained in the residue. Aliquots from the partially evaporated and concentrated filtrate were then evaporated in planchets and counted in an end-window G.M. counter.

The results have been expressed as the concentration of the isotope per unit water in the plant (fresh weight—dry weight) relative to the concentration of the isotope in the substrate.

(c) Weather Conditions

The experiment was conducted in a heated greenhouse exposed to natural light. During the experimental period overcast conditions prevailed on each day, and the maximum temperatures from June 27 to July 1 were 27, 23, 27, 26, and 25°C respectively. The minimum temperatures did not fall below 15°C, the basal temperature for the greenhouse. Relative humidity ranged between 60–80% over the period.

III. RESULTS

In Figures 1, 2, and 5, plant responses to the various osmotic substrates are shown in terms of relative turgidity, water potential (DPD), water content, osmotic potential, transpiration, fresh weight, and dry weight. In each diagram the responses to the inorganic solutes are shown on the left side and to the organic solutes on the right side; also the sampling occasions are indicated on the linear time scale across the bottom of each diagram. Where applicable differences required for significance at the 1 and 5% levels are shown. In every case the osmotic treatments were imposed at the beginning of the experimental period depicted and were removed immediately after sample 4. This initial phase of the experiment is subsequently referred to as stage 1 and the remainder of the experimental period as stage 2.

A photographic record of the progressive changes in appearance is shown in Plates 1 and 2. Following the imposition of the osmotic substrates, the plants in all the high concentrations wilted severely (Plate 1, samples 0 and 1). Slight visible wilting also occurred in the low-concentration treatments, but when sample 1 was taken 75 min after the beginning of the experiment, apparent recovery in these treatments had occurred in all except S_1 . Subsequent visible recovery of all treatments except M_2 proceeded rapidly (Plate 1, samples 2 and 3) and appeared complete 28 hr after the commencement of the experimental period. At this time sample 4 was taken and the osmotic substrates removed, even though M_2 remained severely wilted. Subsequent recovery in M_2 was gradual (Plate 2, samples 5–7) but appeared complete at sample 7, 48 hr later.

These visual responses at first appear to be contradicted by the results of the relative turgidity and DPD determinations (Figs. 1A and 1B), since, after the initial loss of turgor, these quantities did not show significant recovery during stage one in any treatments, and in M_2 continued to deteriorate. However, while the DPD's were held at approximately the level of the external osmotic substrates, it can be seen from Figure 1C that complete recovery in water content occurred in the potassium nitrate, sodium chloride, and sucrose treatments, and this was presumably due to solute absorption and a consequent increase in internal osmotic potential and hence in volume and turgor pressure. In the mannitol treatments the recovery in volume was incomplete by sample 4, suggesting much reduced solute entry.

The lack of recovery in relative turgidity, while partial or complete recovery occurred in water content, likewise appears attributable to solute uptake, since this would result in an increase in internal osmotic potential and an associated increase in water content, even though the DPD of the tissue remained at the level of the

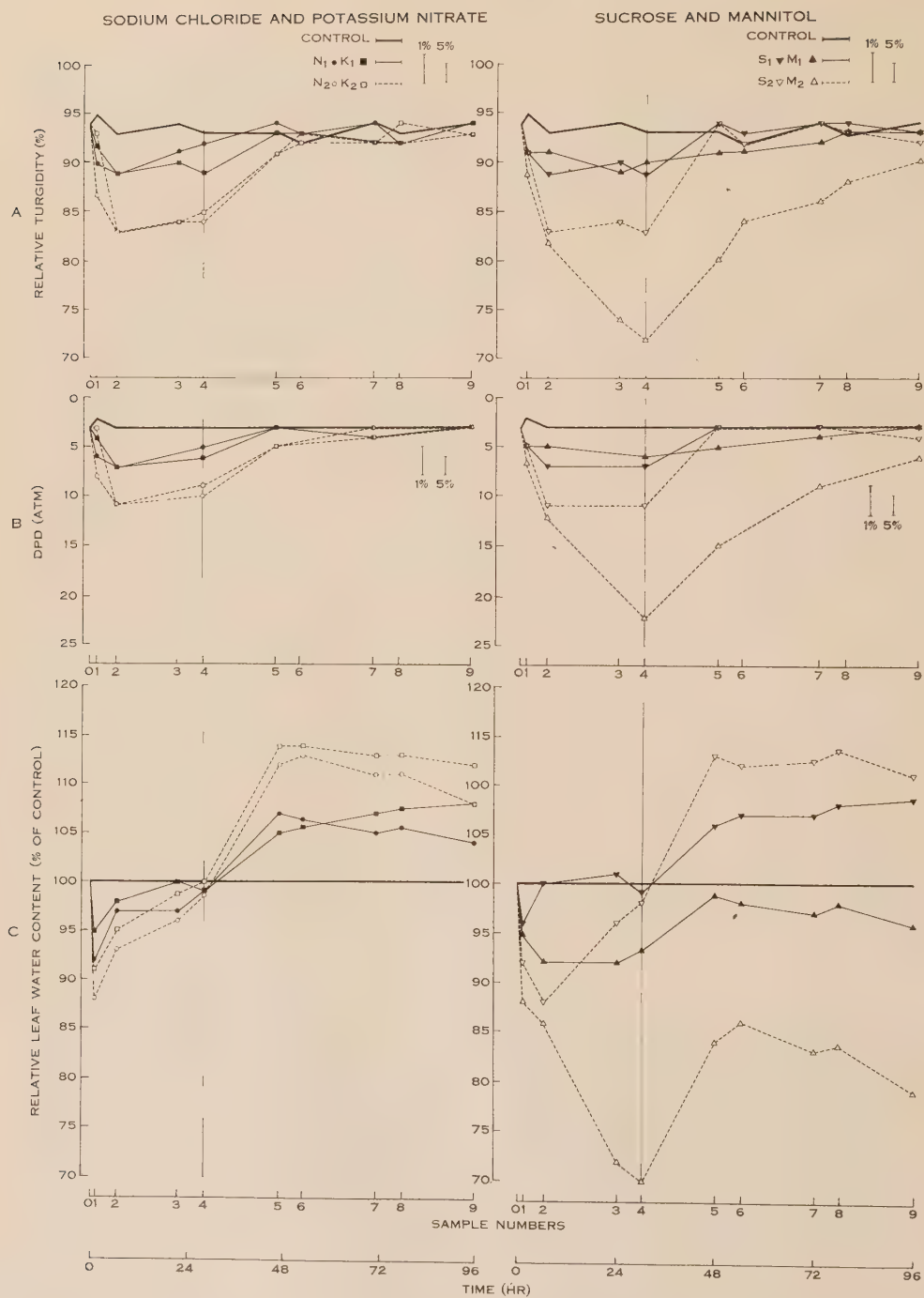


Fig. 1.—Changes during the experimental period in: *A*, leaf relative turgidity; *B*, leaf diffusion pressure deficit (DPD); and *C*, relative leaf water content expressed as fresh weight minus dry weight of treatment leaf tissue relative to that of control.

external substrates. Thus at sample 4, although some treatments had regained their original volume, the fact that all plants still had a DPD equivalent to that of the external solution resulted in the expansion of leaf disks when floated on water. The relative turgidity so measured presumably reflected the additional expansion which occurred before the DPD of the disks was reduced to that of water.

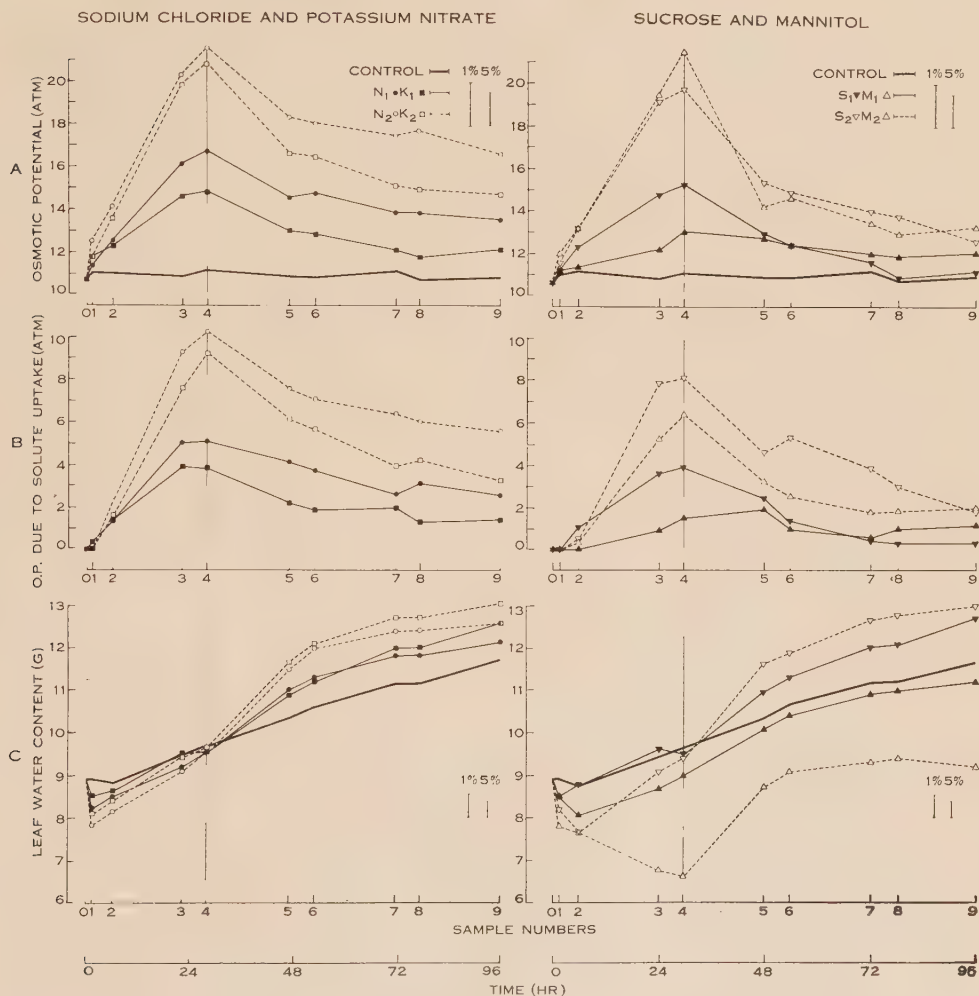


Fig. 2.—Changes during the experimental period in: *A*, total osmotic potential of expressed leaf sap; *B*, total osmotic potential minus osmotic potential due to decreased water content; *C*, leaf water content, expressed as fresh weight minus dry weight.

The response patterns of the plants in stage 2 supports this explanation. Except in M_2 , increase in leaf water content relative to that of the control is seen to be only slightly greater than the initial decrease during stage 1. At the same time relative turgidity and DPD values returned to the control level. In the potassium nitrate, sodium chloride, and sucrose treatments this resulted in an increase of

water content above that of the control, but in M_1 the increase served to bring the water content back to the control level. In M_2 , no compensatory increase in volume occurred even though the recovery pattern showed a close relationship between DPD and relative turgidity. Recovery in M_2 continued throughout the experimental period and appeared visibly complete, but the slow response would appear to indicate tissue injury during stage 1 and complicates data interpretation.

The data of Figure 2 support the hypothesis of solute uptake invoked to explain the data of Figure 1, and indicate substantial increases in internal osmotic potentials

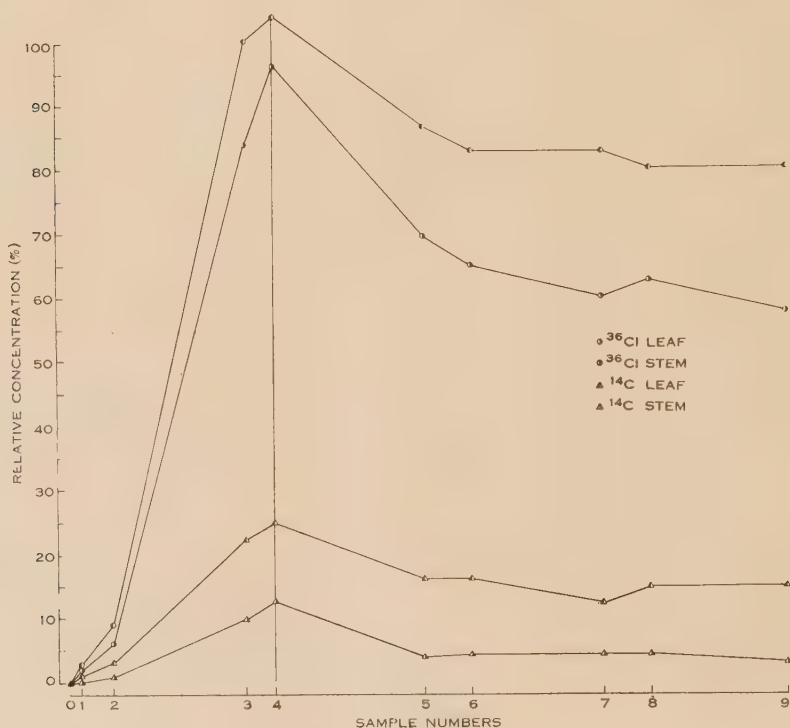


Fig. 3.—Concentration of ^{14}C and ^{36}Cl in the leaves (laminae only) and stems (including petioles) of the high-concentration mannitol and sodium chloride treatments, expressed as a percentage of the isotope concentration in the substrate. Values for samples 5–9 inclusive are given relative to the substrate concentration at sample 4.

during stage 1. From Figure 2A it is apparent that the osmotic potentials increased rapidly in all treatments as soon as the substrates were imposed and, except in M_1 , reached levels which exceeded the control by approximately the same amount as those of the imposed substrate treatments exceeded the base culture solutions.

An increase in osmotic potential can follow the absorption of osmotically active solutes from the substrate, metabolic changes in materials already in the plant, or reduction of internal plant water content. In order to evaluate the contribution of the latter factor, Figure 2B has been constructed by subtracting the osmotic

potential which can be attributed to changes in internal water content (measured as leaf fresh weight—leaf dry weight) from the total osmotic potential. This procedure indicates that the marked reduction in water content in M_1 and M_2 was responsible for about 30 and 40% respectively of the total increase observed. In the other treatments the contribution due to reduced water content was insignificant, since these values were the same as the control by the end of stage 1.

The initial marked increase in internal osmotic potential during stage 1 (due to factors other than dehydration) in the sodium chloride, potassium nitrate, and sucrose treatments, compared with the relatively small increase in the mannitol treatments, suggests that there was greater absorption of osmotically active solutes in the former treatments. In Figure 3, data from the isotopically labelled N_2 and M_2 treatments supports this impression and provides the interesting result that in the case of N_2 , the concentration of ^{36}Cl in the leaves (laminae only) and stems (including petioles) was approximately equal to the concentration in the substrate by the time sample 4 was taken. In the case of M_2 , uptake was much slower and at sample 4 the concentration of ^{14}C in the leaves of the plant was only 25.4% of the substrate concentration. The final concentration in the stems was also much lower (8.3% of substrate concentration) than that of ^{36}Cl . In both the chloride and mannitol treatments initial concentrations developed relatively more rapidly in the leaves than the stems.

After the removal of the osmotic substrates the osmotic potential values declined, but only in the case of S_1 was the control level reached by the end of the experiment. Decline in osmotic potential could have been due to metabolism of absorbed solutes, increase in volume of water in the plant, or loss of solutes to the substrate. In Figure 2C, the primary data for leaf water content is plotted for the different treatments. After the removal of the substrates it is apparent that there was a marked increase in water content in all treatments, and that the relative increase was most marked between samples 4 and 5. These results indicate a similar pattern to that of the decline in osmotic potential and suggest that volume was the primary factor involved. In Figure 4 data for water content, osmotic potential, and isotope concentration during stage 2 are plotted for all treatments relative to the values at sample 4. This confirms the close relationship between the three quantities, but indicates a general tendency for change in water content to exceed the change in osmotic potential.

During stage 2 it is of interest to note from Figure 3 that the concentration of ^{36}Cl in the stems dropped by approximately 40% and the concentration in the leaves by about 25%. This evidence of increased mobility of the material in the stems was more apparent in the mannitol treatments in which the stem concentration dropped to about one-quarter of its value at sample 4. In the leaves the ^{14}C concentration declined by about 40%. In both cases the reduction in concentration in the leaves would appear, from Figure 4, to be primarily due to the increase in the volume of water in the plant, but the marked reduction in stem figures suggests that migration of the ^{36}Cl and ^{14}C from the stems was also operative.

Although it was thought unlikely, the possibility of leakage back to the standard substrates was checked by determining the ^{14}C and ^{36}Cl concentrations in the sub-

strates on the sampling occasions 5–9. Leakage of ^{14}C was found to be negligible, (the counts not differing significantly, at $P = 0.05$, from background). In the case of ^{36}Cl , leakage increased progressively but was so small that the total amount of chloride represented would not have reduced the osmotic potential in the leaves of the plant by more than 0.2 atm.

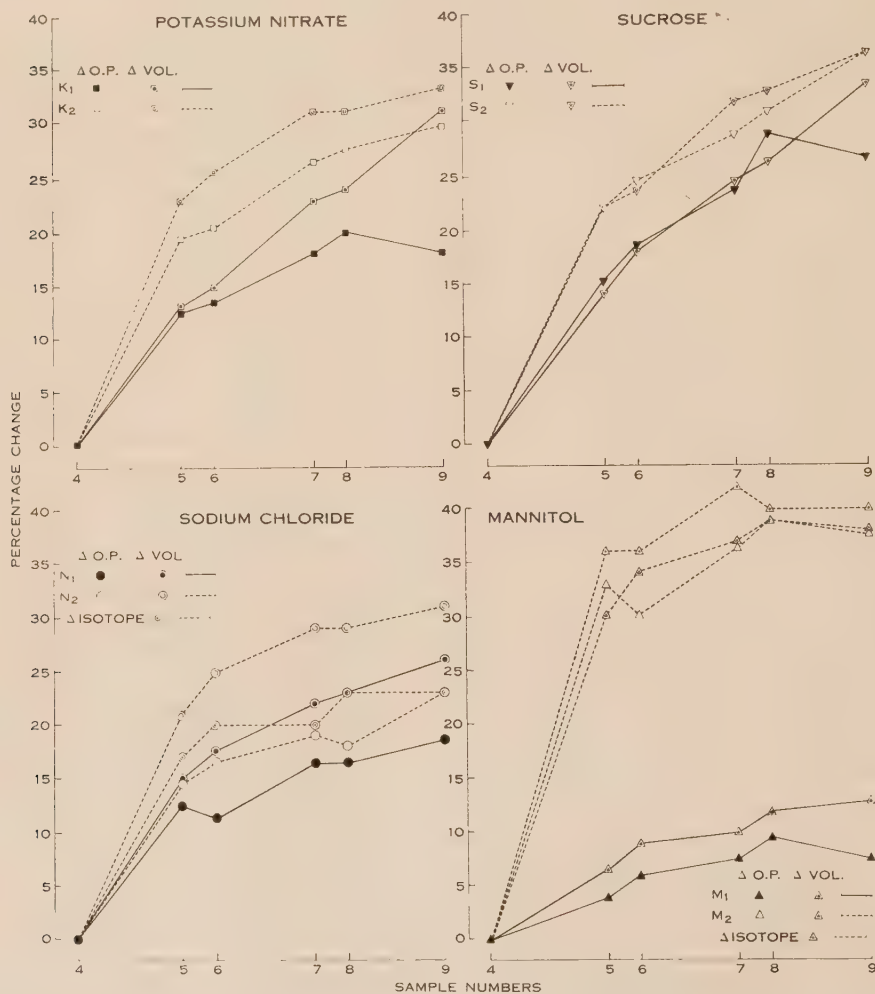


Fig. 4.—Changes in internal osmotic potential (Δ O.P.), isotope concentration (Δ isotope), and leaf water content (Δ volume) during stage 2, relative to values at sample 4.

The influence of the osmotic treatments on subsequent plant activity was difficult to determine during such a short experimental period but in Figure 5 data on transpiration and growth (measured as increase in dry weight) are presented.

The transpiration data reflect the marked reduction in water absorption at the beginning of the experiment and the rapid increase in water absorption as the plants regained water content and turgor. In all the low-concentration treatments

except M_1 , the transpiration values were close to those of the control by sample 4. In all the high-concentration treatments except M_2 , rapid recovery occurred during stage 1 but the values were still significantly lower than the control at sample 4.

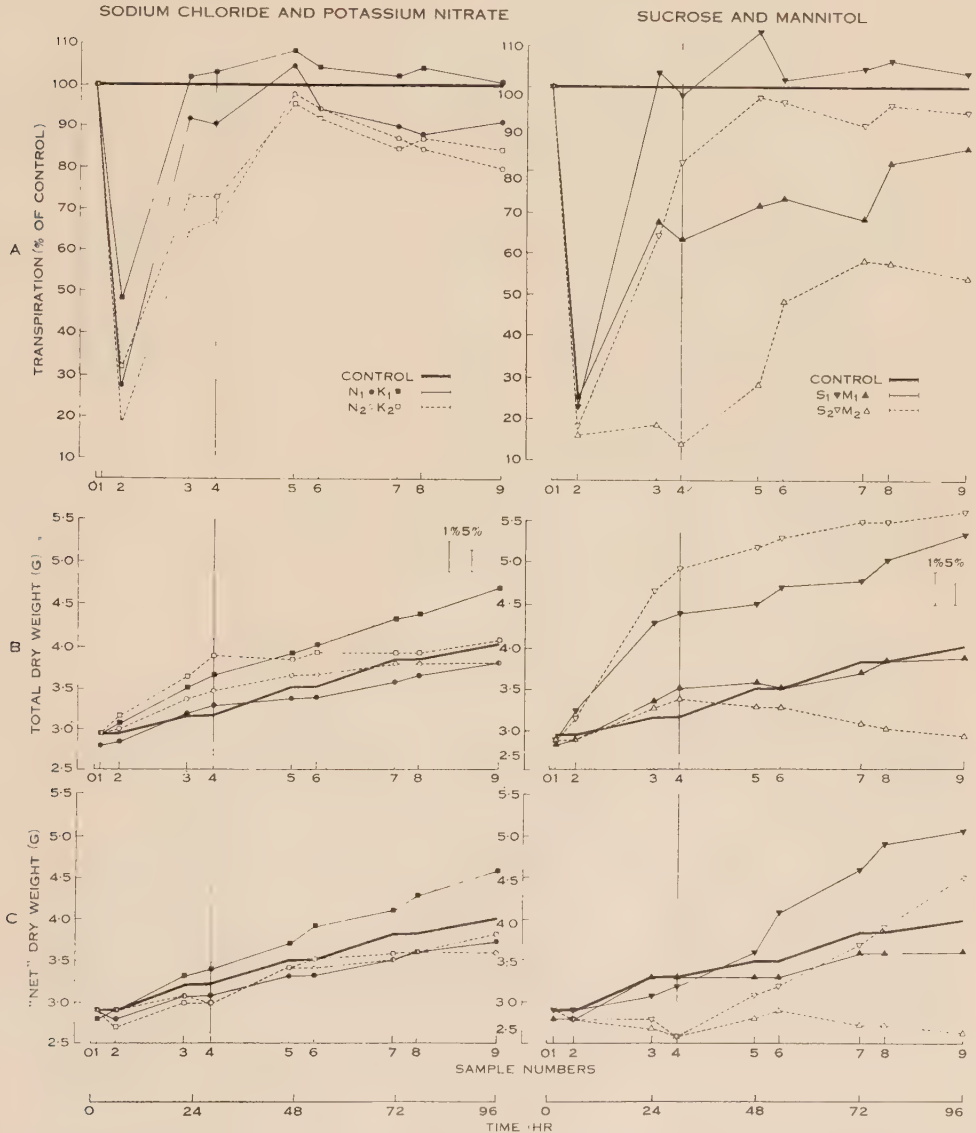


Fig. 5.—Changes during the experimental period in: A, transpiration, expressed as percentage of the control; B, total dry weight per plant; C "net" dry weight (for explanation see text, p. 530).

The lowest values recorded, approximately 20% of the control, may well be associated with complete stomatal closure since this low value was maintained, in the M_2 treatment, until the removal of the osmotic substrates. After sample 4, transpiration

values in all treatments increased further, reaching levels significantly higher (at $P = 0.05$) than the control in K_1 and S_1 . In these treatments transpiration values did not fall below the control for the remainder of the experiment, but in all other treatments final values were below the control and the transpiration of the high-concentration treatments declined slightly towards the end of the experiment.

The dry weight data of Figure 5*B* indicate that, during stage 1, increase in dry weight in some treatments was much more rapid than the control and in no treatment was slower than the control. However, this result was undoubtedly influenced by the fact that stage 1 was the period of rapid absorption of osmotically active solutes and the increases cannot be regarded entirely as due to photosynthetically induced dry weight increase. Subsequently the rate of increase appeared similar to the control in all treatments except K_1 and S_1 , in which it was more rapid, and in M_2 , in which it was markedly depressed.

In order to give an indication of photosynthetically induced dry weight increase Figure 5*C* has been included. This diagram has been constructed by subtracting, from the data of Figure 5*B*, amounts equivalent to the weight of treatment solute which is represented by the observed increase in osmotic potential. (Data for this calculation were based on Figure 2*B* and excluded dehydration as a factor causing increased osmotic potential.) This procedure involves the assumption that only the added solute, in each treatment, contributed to the increase in osmotic pressure. Although an oversimplification, this is of value in that it indicates that "net" growth rates were significantly depressed by the osmotic substrate treatments in N_2 , M_2 , and S_2 . The extent of the apparent depression in S_2 and M_2 is much greater than anticipated and would appear to provide good evidence that only part of the observed increase in osmotic potential in these treatments was due to absorption of sucrose and mannitol respectively. In stage 2 the data of Figure 5*C* are in good agreement with those of Figure 5*B*, although they suggest that subsequent growth in both sucrose treatments was significantly more rapid than the control.

All treatments were observed for 1 month beyond the end of the experimental period. At this time the general pattern of growth depicted in Figure 5 was still generally valid, although both sodium chloride and mannitol treatments were smaller than the control and the potassium nitrate and sucrose treatments did not appear significantly different. In N_2 and M_2 the leaves which were on the plants at the end of the experimental period did not fully regain the colour and vigour of the control, and the new leaves were smaller and the growth rate slower. Radioautographs of all leaves at this time indicated some migration of chloride and mannitol into the sixth and seventh leaves but virtually no evidence of radioactivity in subsequent leaf development.

IV. DISCUSSION

At the present time there is general agreement in the literature that the exposure of plants to substrate solutions or soils containing soluble salts (or other osmotically active solutes) in excess of those required for normal growth usually results in decreased water absorption, disturbed nutrient uptake and metabolism, and reduced growth. It is also generally agreed that when soluble salts are the osmotically active agents the effects on plants are of two main types, due in part to the direct osmotic

effects of increased soil or substrate water stress and in part to specific toxic effects of individual ions (Magistad 1945; Hayward and Wadleigh 1949; Bernstein and Hayward 1958).

Evidence for the influence of direct osmotic effects may be found in the numerous experiments conducted with iso-osmotic concentrations of different mineral salts and organic solutes in which the degree of inhibition of growth has effectively been the same regardless of the solute employed (Eaton 1941; Long 1943; Magistad *et al.* 1943; Gauch and Wadleigh 1944; Hayward and Spurr 1944*a*, 1944*b*). It is also supported by those studies concerned with the influence of increasing total soil moisture stress on plant growth in which the effect has been the same regardless of whether the total stress was composed mainly of soil moisture tension or mainly of an osmotic potential in the soil solution (Ayers, Wadleigh, and Magistad 1943; Wadleigh and Ayers 1945; Wadleigh, Gauch, and Magistad 1946).

In order for the osmotic potential of the soil or substrate solution to contribute to the water stress around the roots in the same manner as soil moisture tension, it would appear to be necessary for the plant to act as an ideal osmometer so that osmotically active solutes in, or added to, the substrate remain outside the plant-water system and induce an additional internal water stress of equal magnitude to their own osmotic potential. If, on the other hand, the solutes are diffusible into the plant-water system it would seem that a water stress of equal magnitude could still be induced, but that the degree to which it simulated soil water tension would vary with the amount of solute absorbed and the degree to which the internal osmotic potential was consequently increased.

It is apparent that in the strictest sense the ideal osmometer concept is not valid since mineral nutrients must be absorbed by the plant. However, Bernstein and Hayward (1958) have suggested that a layer or layers of plant cells may exist in the root defining the limits of the free space. These cells would transmit water into the plant water system along normal DPD gradients but would restrict the absorption of certain ions, thereby building up, in the outer layers, a concentration which would prevent further diffusion of a particular ion into the plant and may even promote outward diffusion to the root medium if the concentration became high enough. The existence of a barrier such as this, which can offer high resistance to the passage of ions across the symplast of the plant roots, has also been indicated by the work of Scott Russell and Shorrocks (1959) and could probably effectively control ion and solute absorption. However, it would seem that such a barrier would still have to prevent the absorption of solutes, other than those required for normal growth and metabolism, to effectively simulate soil water tension effects in the induction of internal water stress.

If such a barrier exists and is differentially permeable as far as osmotic solutes are concerned, one would envisage that the water relations of plants, exposed to substrates in the same manner as in the present experiment, would respond as shown in Figure 6*A*. If, however, no such obstacle exists and the solutes are freely diffusible into the plant, but not freely diffusible back to the substrate, one would expect the water relationships to respond as shown in Figure 6*B*.*

* The terms "diffusible" and "non-diffusible" are used in this paper for convenience and are not intended to imply that diffusion is the only, or primary, factor involved in solute entry.

From the results presented earlier it is apparent that the response pattern of the plants to potassium nitrate, sodium chloride, and sucrose followed closely that suggested in Figure 6*B*. Mannitol, on the other hand, caused a response pattern intermediate between the two type situations as might be expected from its reduced

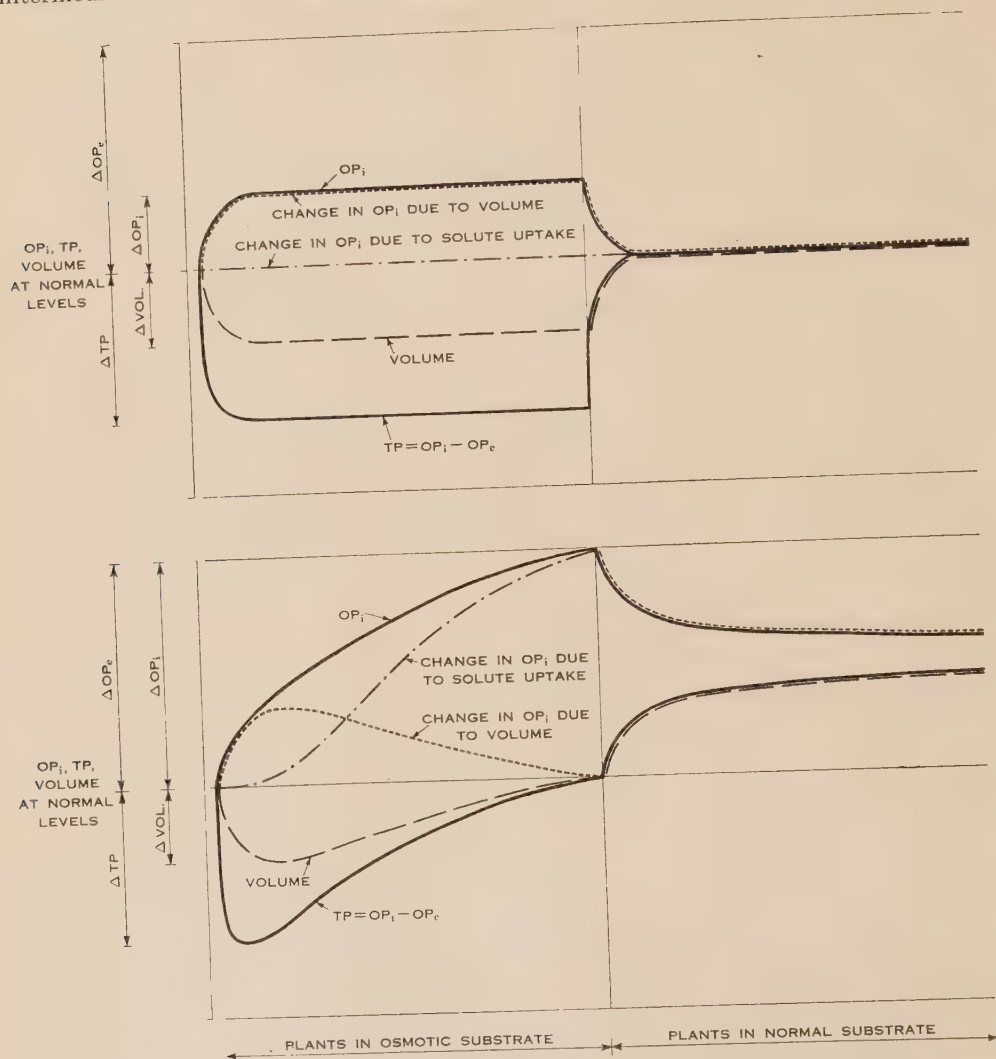


Fig. 6.—Diagrammatic representation of expected changes in internal water relations of plants exposed to a non-diffusible osmotic substrate (A), or exposed to an osmotic substrate in which solutes are freely diffusible into the plant but non-diffusible from plant to substrate (B). TP and OP_i represent the turgor pressure and osmotic potential, respectively, and OP_e represents the increase in substrate osmotic potential induced by the addition of osmotically active solutes.

diffusibility. In both cases it is apparent that the plant DPD, represented by the difference between the internal osmotic potential and turgor pressure, was maintained at approximately the level of the substrate osmotic potential. However, the

response patterns of relative turgidity, water content, turgor pressure, and internal osmotic potential differed in several important respects and it is consequently of value to discuss these relationships in more detail.

(a) Significance of Water Content and DPD Changes

Of particular interest is the fact that, during stage 1, visual recovery of turgor occurred in the potassium nitrate, sodium chloride, and sucrose treatments without compensating recovery in DPD. It appears that the type situation of Figure 6*B* provides a valid explanation of this phenomenon and suggests a close, but expected, relationship between tissue water volume and turgor pressure. Thus, initially, turgor pressure and volume decreased, from the direct osmotic effects of the imposed substrates, until the DPD approximately equalled the external osmotic potential. This was associated with severe wilting of plants in the high-concentration treatments. Subsequently, although the DPD remained at this level, solute entry proceeded and internal osmotic potential, turgor pressure, and volume increased until the latter two quantities regained their original values. At this point it is apparent that the normal appearance of the treatment plants was to be expected, even though the tissue was not turgid; the reduction in turgor pressure below the value at full turgor being equivalent in magnitude to the imposed external osmotic potential.

In the case of non-diffusible substrates, it is suggested in Figure 6*A* that the initial response should be similar to that just described, but the absence of solute entry should preclude any increase in internal osmotic potential apart from that due to reduced volume. Thus, turgor pressure and volume would remain below the original levels and a direct osmotic stress would be imposed on the plants.

It appears that the mannitol treatments responded in a manner between the two type situations in that, although some solute uptake occurred, there was no significant recovery in volume by either M_1 or M_2 during stage 1. In M_2 , not only volume, but also relative turgidity continued to decline steeply, and DPD continued to increase. This was unexpected since it was anticipated that DPD should not increase to a value much beyond the substrate osmotic potential and that any absorption of mannitol would result in an increase in volume. Consequently, it appears the continued decline in the internal water balance was due to toxic side effects of the high concentration of mannitol. The subsequent response pattern of the plants in the M_2 treatment supports this view. Even so, it is of interest to note the close relationship between DPD and internal osmotic potential in M_2 as leaf volume and relative turgidity levels continued to decrease; the DPD values at samples 2 and 4 being 14 and 22 atm and the osmotic potential values 13.2 and 21.0 atm respectively. This provides evidence that negative wall pressure was not of significance at stress levels greater than those prevailing when the DPD first rose to the level of the internal osmotic potential.

In all cases other than M_2 , DPD increased to values of approximately 7 atm in the low-concentration treatments and 12 atm in the high-concentration treatments and were maintained at these levels throughout stage 1. When tissue segments are floated in aqueous solutions containing diffusible solutes, it is normally assumed that the tissue DPD equilibrates with the osmotic potential of the solution. However,

Weatherley (1955), in a study of sucrose uptake by floating leaf disks, found a higher DPD in the disks than in the surrounding solution and concluded that the sucrose was absorbed actively and that water entered the tissue passively along a diffusion pressure gradient. It is possible that a similar mechanism operated in the present instance, although the DPD in the control plants averaged 3 atm and it could be argued that the imposition of the osmotic substrates merely served to increase the treatment DPD's by similar amounts.

(b) Characteristics of the Changes in Internal Osmotic Potential

From Figures 2 and 3 it is apparent that internal osmotic potentials increased rapidly in all treatments as soon as the osmotic substrates were imposed. Initially, this appeared to be due primarily to the direct effect of dehydration on the concentration of the tissue fluids since the data of Figure 3 indicate that little ^{36}Cl and ^{14}C was absorbed in the first hour. Subsequently, however, solute entry proceeded rapidly, and it is apparent that (from Fig. 2B) by the end of stage 1 virtually all of the increase in osmotic potential in the non-mannitol treatments could be attributed to absorption of osmotically active solutes from the substrate or to metabolic changes within the plant.

Volume recovery and increase in osmotic potential was most rapid in the sodium chloride and potassium nitrate treatments and probably reflected the more rapid uptake of these inorganic solutes than of sucrose. This is in accord with other studies (Beck 1927) even though Weatherley (1955) has shown that sucrose uptake by leaf tissue can result in a virtual doubling of the dry weight within 24 hr. In the case of sodium chloride it is apparent from Figure 3 that the increase in osmotic potential was almost entirely due to uptake of chloride and probably also of sodium ions. This is in agreement with other studies (Eaton 1942; Black 1956) in which chloride concentrations in the plant have reached values as high or higher than in the substrate. No evidence is available to indicate to what extent absorption of KNO_3 or of potassium or nitrate ions was responsible for the osmotic potentials developed in the K_1 and K_2 treatments, but, because of its high molecular weight, indirect evidence from Figure 5C indicates that, in the case of sucrose, only part of the increase in osmotic potential was due to sucrose uptake and the remainder was presumably due either to stimulated uptake of inorganic solutes, polysaccharide breakdown, or conversion of the absorbed sucrose to hexoses.

Compared with the other osmotic substrates, the relative contribution of absorbed solutes to the total internal osmotic potential was much smaller in the mannitol treatments. From Figures 2A and 2B it can be seen that about 40% of the M_2 value at sample 4 could be attributed to the direct effect of reduced water content and in M_1 the figure was approximately 30%. However, the ^{14}C absorption data indicate that the concentration of ^{14}C in the M_2 plants at sample 4 was only 25% of the substrate concentration. This suggests that a significant proportion of the observed osmotic potential may have been due to absorption of other solutes from the substrate, or to breakdown of an absorbed polymer, as suggested by Thimann, Loos, and Samuel (1960). The data of Figure 5C, interpreted previously as in the case of sucrose, support this view.

It is of interest that these authors found little evidence of mannitol uptake by potato tissue disks, except in the free space. It is possible that the relatively substantial uptake observed in M_2 may have been associated, to some extent, with tissue injury.

At the end of stage 1 it is of interest to examine the differentials between the osmotic potentials in the leaf tissue of the treatment plants and in the various substrates, in comparison with the differential between the osmotic potential in the control plants and in the base nutrient solution. These data are listed in Table 1 and demonstrate that in all except the low-concentration mannitol treatment the differential was maintained in all treatments within 1 atm of the control value.

TABLE 1
OSMOTIC POTENTIALS OBSERVED IN ALL TREATMENTS AT SAMPLE 4 COMPARED WITH SUBSTRATE POTENTIALS

Substrate	Low-concentration Treatments				High-concentration Treatments			
	Level	O.P. of Substrate (atm)	O.P. of Expressed Sap (atm)	Difference (atm)	Level	O.P. of Substrate (atm)	O.P. of Expressed Sap (atm)	Difference (atm)
Potassium nitrate	K_1	5.7	14.9	9.2	K_2	10.7	20.8	10.1
Sodium chloride	N_1	5.7	16.7	11.0	N_2	10.7	21.6	10.9
Mannitol	M_1	5.7	13.0	7.3	M_2	10.7	21.0	10.3
Sucrose	S_1	5.7	15.2	9.5	S_2	10.7	19.7	9.0
Control		0.7	10.9	10.2		0.7	10.9	10.2

Although this has previously been demonstrated by Eaton (1942), and was to be expected if the response pattern conformed to the "freely diffusible" model, it provides strong evidence of the degree to which absorption of osmotically active solutes can lead to the re-establishment of turgor pressure and tissue volume at normal levels.

In both mannitol treatments it was expected that the differentials would be less than with the freely diffusible substrates. Although this applied in M_1 it was not apparent in M_2 presumably because of the physiological damage to the plants in this treatment. Thus the M_2 differential can be regarded, not as representative of an undamaged plant in a stable plant-substrate water balance, but rather as representative of a plant in which the water balance was progressively deteriorating as long as it was exposed to an osmotic substrate. To this extent it appears to have been fortuitous that the differential at sample 4 was so similar to those of the other treatments.

Following the removal of the osmotic substrates the mannitol treatments responded almost as anticipated from Figure 6*A* and those in the potassium nitrate, sodium chloride, and sucrose treatments as anticipated from Figure 6*B*. In all cases a marked recovery occurred in relative turgidity and DPD, these quantities reaching levels not significantly different to the control within 24 hr (except in the case of M_2 where recovery was still incomplete at the end of the experimental period). Associated with these responses were substantial increases in volume (as water content) and reciprocal changes in internal osmotic potential.

The decline in osmotic potential and isotope concentration during stage 2 (Figs. 2, 3, and 4) could have been caused, apart from increases in volume and consequent dilution, by either leakage of osmotically active solutes back to the base nutrient solutions or by their metabolic incorporation. However, leakage of ^{14}C and ^{36}Cl was found to be almost negligible in the high-concentration mannitol and sodium chloride treatments and the work of Long (1943) in a similar experimental sequence suggests that leakage in the potassium nitrate and sucrose treatments would also have been very small.

Some idea of possible metabolic effects can be gained from Figure 4 in which a general tendency for volume changes to exceed changes in osmotic potential can be seen. This suggests that renewed growth of the plants in stage 2 was possibly associated with renewed mineral uptake from the culture solution and accumulation of osmotically active photosynthetic products. Such processes would be expected to contribute to both tissue expansion and osmotic potential and would tend to cause a trend of the type observed. The fact that this effect was most marked in the potassium nitrate and sucrose treatments, in which most rapid growth occurred, would support this contention as does the evidence that decline in isotope concentration in M_2 and N_2 was related to volume more closely than was osmotic potential.

The metabolic incorporation of the osmotic substrate solutes into non-osmotic compounds would be expected to result in reduced osmotic potential relative to volume except in the case of sucrose, since the normal fresh weight/dry weight ratio is of the same order as the water volume/sucrose ratio expected at the prevailing levels of osmotic potential. It is doubtful if significant incorporation of this type occurred in the non-sucrose treatments and the data of Figure 4 would, in summary, appear to provide satisfactory evidence of an effectively reciprocal relationship between changes in osmotic potential and volume in stage 2.

(c) *Relative Turgidity and Water Content Relationships*

The relative turgidity changes were associated, in M_1 and M_2 , with water content and turgor pressure recovery towards the level of the control plants whereas in other treatments water content, which was already at the control level, increased with a proportional increase in turgor pressure as would be expected from Figure 6*B*. In all treatments except M_2 it is of interest to observe that the relative turgidity levels remained fairly constant during stage 1. This suggests that the reduction in volume during the initial period of reduced turgor measured, in the case of tissue disks, by the amount of water uptake during the course of the relative turgidity

determination, was equivalent to the increase in volume which occurred when disks taken at sample 4 were floated on water. In turn, this implies that the volume of the leaf tissue as a whole should have responded similarly when the plants were returned to base culture solution. This is generally borne out in the leaf water content data of Figure 1C, although the increase in water content in stage 2, relative to the control, was slightly greater than the initial decrease in stage 1 and the generally more rapid growth in the control plants compared with most treatments would, if anything, tend to enlarge this difference.

It was thought that a too-short period of floating during the determinations could have contributed to the apparent constancy of relative turgidity during stage 1 if water uptake and tissue expansion became slower as tissue volume regained its original value. Data relevant to this point were available from samples 1, 4, 5, 7, and 9 in which a 24-hr period of floating was adopted in addition to the standard 4-hr period. These determinations revealed that, in the present experiment, although there was a tendency for more water uptake, after the standard 4-hr period, in the high-concentration treatments compared with the low, the slopes of the different uptake curves in each treatment, from the various sampling occasions, did not differ significantly after the standard 4-hr period. Leakage of solutes from the floating leaf disks to the surrounding water was also investigated as a possible contributing factor by measuring the electrical conductivity of the water before and after floating and the dry weight of dissolved solids after the water had been evaporated. Although some leakage occurred the quantities were not sufficient to significantly affect the relative turgidity values obtained.

(d) *Transpiration and Growth*

The data of Figure 5 on transpiration and growth warrant comment on several points. It is of interest that reduction of water absorption was closely related to turgor pressure and water content and once recovery of these quantities commenced water absorption rapidly returned to values close to normal. This is similar to the results of Renner (1912) and Montfort (1920) but is at variance with findings by some other workers (Eaton 1941; Hayward and Spurr 1944*b*) in long-term experiments where continued reduction of water absorption was observed as long as the osmotic treatments remained. In such cases Hayward and Long (1941) have noted damage to the absorbing zone of the roots and it is probable that a similar effect would have been observed in the present study had the treatments been prolonged. The present results provide good support for transpirational control of absorption, since the degree of stomatal closure could be expected to be generally proportional to the turgor pressure in the leaf tissue. In stage 2 of the experiment transpiration in excess of the control was observed in K_1 and S_1 treatments. A similar response was observed by Rybin (1923) but in this instance it appeared to be due simply to the enhanced growth and greater plant size in these treatments. By comparison, transpiration in all the high-concentration treatments declined towards the end of the experiment. Although this possibly indicated toxic side effects, the tendency to relatively slower growth and smaller plants in these treatments provides a simpler explanation.

The data on dry weight changes in Figure 5*B* are difficult to interpret during stage 1 because of the considerable quantities of osmotic solutes absorbed. However, from the data of Figure 5*C* it appears doubtful if significant reductions occurred. During stage 2, when no further uptake of this type was possible, increase in dry weight appeared slower in all the high-concentration treatments relative to the low, confirming the results of other studies (Bernstein and Hayward 1958). From Figure 5*B* two treatments (K_1 and S_1) appeared to have more rapid rates of increase than the control due probably to the fact that during stage 1 these substrates provided low concentrations of solutes which were also plant nutrients. From Figure 5*C* it appears that S_2 also increased more rapidly than the control in stage 2. Since this treatment was characterized by a rapid reduction in osmotic potential it is probable that some of the absorbed sucrose was metabolized by the plants. Only in M_2 did dry weight decline in stage 2.

(e) *Concluding Remarks*

In conclusion it seems appropriate to emphasize that the plant responses to the potassium nitrate, sodium chloride, and sucrose treatments closely paralleled those to be expected if the added solutes were assumed to be freely diffusible into the plant. The responses to mannitol, while similar in some respects to those of a model based on non-diffusibility, differed to the extent that some mannitol was absorbed and the plants, to this degree, responded in an intermediate manner. These results suggest that Walter's (1955) premise, that if the substrate solutes are diffusible the osmotic potential is balanced by solute entry, is valid to the extent that turgor pressure and tissue volume returned to their original values after an initial period of adjustment. However, it is apparent that DPD did not return to normal and instead was maintained at the level of the imposed substrate osmotic potential. Hence the plants were not turgid and the turgor pressure appeared to be depressed below its value at full turgor by an amount equal in magnitude to the osmotic potential of the substrate. Thus, although the osmotic effect of diffusible substrates appears to be real in the sense that a water stress is induced in the plant, it is not strictly analogous to the effect of soil water tension since the osmotic potential and turgor pressure levels are displaced.

It seems that the present results are of value in interpreting the extensive literature dealing with the effects of osmotic substrates on plant growth since the *absolute* reduction in turgor pressure below the internal osmotic potential appears to be equal in magnitude to the imposed substrate potential, regardless of whether this is due to diffusible or non-diffusible solutes, and it therefore appears logical to expect that growth inhibition should increase with increase in substrate concentration. However, the *relative* reduction in turgor pressure decreases proportionally with the amount of solute absorbed and hence with the increase in internal osmotic potential. Thus, when diffusible solutes are involved, it seems that the analogy between water tension effects and osmotic effects becomes less valid as substrate concentration is increased and to this extent the attribution of growth inhibition and other plant responses entirely to direct osmotic effects should be undertaken with caution.

V. ACKNOWLEDGMENTS

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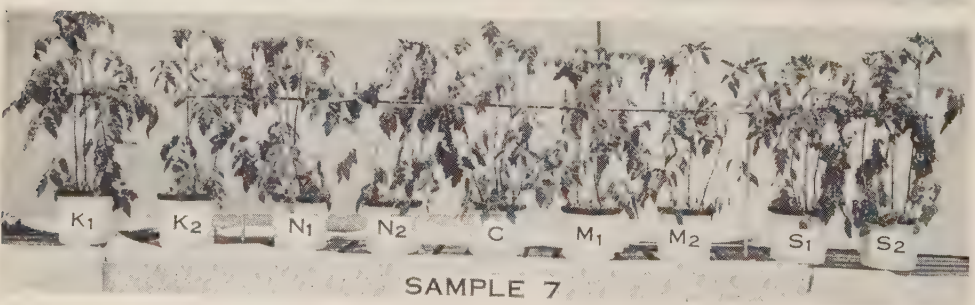
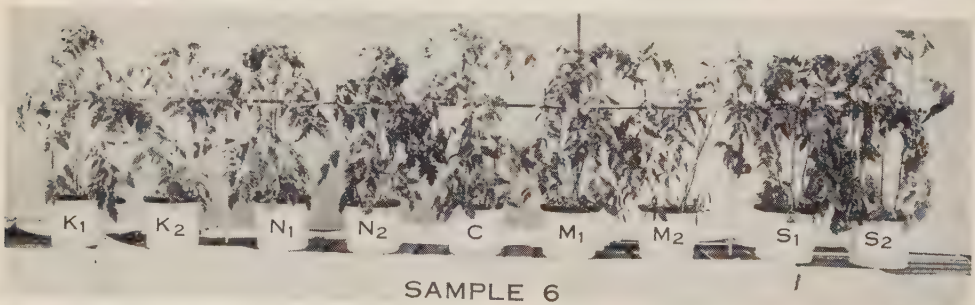
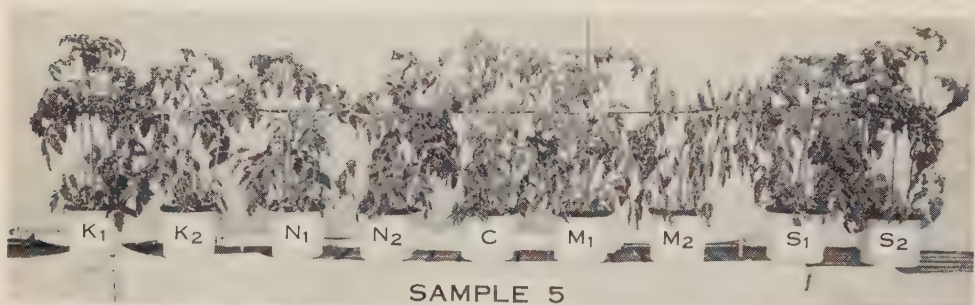
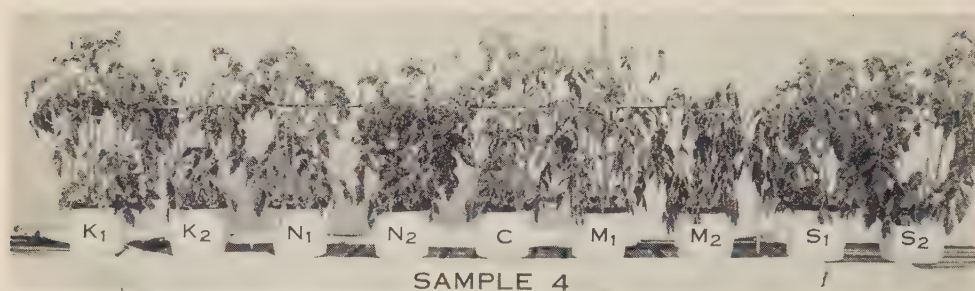
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PLANT-WATER RELATIONS IN OSMOTIC SUBSTRATES



Visual appearance of tomato plants at sample numbers 0-3. Treatments are indicated on the containers, using the same key as in the text. C, control.

PLANT-WATER RELATIONS IN OSMOTIC SUBSTRATES



Visual appearance of tomato plants at sample numbers 4-7. Treatments are indicated on the containers, using the same key as in the text. C, control.

THE ELECTRIC DOUBLE LAYER AND THE DONNAN EQUILIBRIUM IN RELATION TO PLANT CELL WALLS

By J. DAINTY* and A. B. HOPE†

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Summary

The division of free space in plant tissues into "water free space" (W.F.S.) and "Donnan free space" (D.F.S.) is examined in systems which contain electrically charged surfaces separated by various distances. It is suggested that plant cell walls should be described in terms of a system of electric double layers and not by classical Donnan equations. An approximate theory is presented which results in an expression for the equivalent width of D.F.S. in terms of the external concentration but which is independent of the surface charge density.

The theory is applied to experiments on the replacement of calcium by sodium in cell-wall segments of *Chara australis* and to data in the literature on solutions of gum arabic and on jute fibres. The variation in apparent concentration of indiffusible ion in these systems with external concentration is consistent with the double-layer theory and reaffirms that use of classical Donnan theory, which assumes a *homogeneous* charged system, is only permissible in limiting cases.

The double-layer theory leads to a value of 4×10^{-5} C. cm⁻² for the average charge density in cell walls from *C. australis*.

I. INTRODUCTION

Cation exchange between plant cells and an external medium has been observed for many years, but only recently have attempts been made both to put it on a quantitative basis and to establish the site of the initial cation exchange in plant cells. This site has been assumed by some to be the cytoplasm of the cell (containing proteins, lipids, phosphate compounds, etc., with a predominantly negative net charge) and by others to be the cell wall, which contains cellulose organized in microfibrils of various orientations, together with appreciable amounts of "pectins" and "hemicelluloses". The unmethylated form of pectin is polygalacturonic acid, and the hemicelluloses also contain many uronic acid residues, probably mainly glucuronic acid.

The use of preparations of cell walls isolated from large algal cells (*Chara*: Gaffey and Mullins 1958; Dainty and Hope 1959; Dainty, Hope, and Denby 1960; *Nitella*: Diamond and Solomon 1959), and from coleoptiles (Jansen *et al.* 1960), has shown conclusively that the site of the initial cation exchange is the cell wall and, moreover, that the "exchange capacity" can be accounted for by uronic acid, the presence of which has been established by chemical analysis.

In plant cells and tissues a division can be made of the space freely accessible by diffusion from the external medium into "water free space" and "Donnan free

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space" (Briggs and Robertson 1957; Briggs, Hope, and Pitman 1958). "Water free space" (W.F.S.) is that part of the space in which mobile cations and anions are in equal (equivalent) concentration, and corresponds to the extracellular space referred to by workers on animal cells or tissues. "Donnan free space" (D.F.S.) has in the past been assumed, for the purposes of analysis, to be a homogeneous space containing indiffusible or restrained ions and to which the classical Donnan equations relating internal and external ionic activities can be applied.

Considerable error may be involved in the assumption that any fixed part of plant cell walls corresponds to either W.F.S. or D.F.S., as defined. The polysaccharide chains, some of which contain uronic acid residues, are probably separated—to judge by electron-micrographs and by cell-wall permeability—by distances such that the classical Donnan equations would not be applicable, for which reasons have been stated by Overbeek (1956).

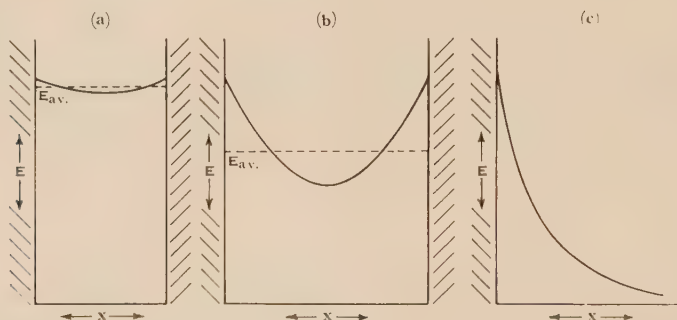


Fig. 1.—Variation in electric potential (E) with distance (x) between charged surfaces: (a) when the surfaces are relatively close together; (b) when the surfaces are further apart; (c) when the surfaces are infinitely separated. E_{av} is the average electric potential.

The argument can be illustrated by Figure 1. This shows the variations in electric potential E with distance (x) between charged surfaces, (a) in what would be an approximately homogeneous Donnan phase; (b) in a more "dilute" system where the potential approaches zero at the points of symmetry; and (c) for a single charged surface.

If the variation in potential between the surfaces is small, as in Figure 1(a), it is reasonable to suppose that the average potential will be related through a Donnan equation to the average concentrations of the cations (\bar{C}_+) and anions (\bar{C}_-); i.e. for this situation:

$$\bar{C}_{\pm} = C_o \{\exp(\mp FE/RT)\} = C_o \{\exp(\mp FE_{av}/RT)\}, \quad (1)$$

where C_o is the concentration of electrolyte, assumed uni-univalent and at some distance from the charged system, and E_{av} is the average potential (e.g. measured by a probe electrode) in the liquid phase of the charged system. F , R , and T have their usual significance. Activities are assumed equal to concentrations throughout.

If the variation in potential is large, as in Figures 1(b) and 1(c), it is not permissible to say that the average of $\exp(\pm EF/RT)$ is equal to $\exp(\pm E_{av} F/RT)$. Since the physical situation in the cell wall is more likely to correspond to Figure 1(b) or 1(c) the question arises: where does W.F.S. end and D.F.S. begin? A theoretical treatment, based on Overbeek's remarks, has been developed to deal approximately with this situation and is described below.

II. THEORY

By use of Poisson's equation relating local electric potential to local volume charge density and Boltzman's statistical law (see equation (1) above), the Gouy-Chapman theory of the diffuse double layer near a uniformly charged plane surface leads to the following equations for the charge density on a surface and for the potential near a charged surface in the presence of an aqueous solution of a uni-univalent electrolyte:

$$\sigma = \sqrt{(\epsilon RT C_o / 2\pi)} [\exp(E_o F / 2RT) - \exp(-E_o F / 2RT)], \quad (2)$$

where σ = surface charge density in coulomb . cm⁻²,

ϵ = the permittivity of the medium [$80/(9 \times 10^{11})$ C.V⁻¹ . cm⁻¹ for water],

R = the gas constant (8.31 J . mole⁻¹ . deg⁻¹),

T = temperature in degrees Kelvin,

E_o = the electric potential of the charged surface, relative to the bulk phase, in volts,

C_o = the concentration in the bulk phase in equiv . cm⁻³,

F = 96,500 C . equiv.⁻¹,

and x , the separation distance in centimetres, is related to E , the potential at that distance by:

$$x = -\sqrt{\{\epsilon RT / 8\pi F^2 C_o\}} \ln\{\{\tanh(EF/4RT)\} / \{\tanh(E_o F / 4RT)\}\}. \quad (3)$$

From these equations local concentrations can be calculated as functions of x . Analogous expressions can be derived for calcium sulphate or similar electrolytes in the bulk phase.

In a negatively charged double-layer system there is no sharp break into a D.F.S. and a W.F.S. — C_+ is greater than C_- from $x = 0$ to $x = \infty$. However, it is possible to calculate the formally equivalent D.F.S., for the system is equivalent to a D.F.S. extending from $x = 0$ to $x = x_D$ (to be determined) and a W.F.S. extending from $x = x_D$ to $x = \infty$. This is illustrated in Figure 2.

In the equivalent D.F.S. the concentration of indiffusible anions will be $\sigma/(F \cdot x_D)$ equiv . cm⁻³ and the concentrations of the diffusible cations and anions C'_+ and C'_- will, *by definition*, obey the Donnan equations:

$$C'_+ \cdot C'_- = C_o^2, \quad (4)$$

and

$$C'_+ = C'_- - \sigma/(F \cdot x_D). \quad (5)$$

Outside the D.F.S. is W.F.S. where the concentration of both ions is C_o . Thus the amount of diffusible anion excluded per cm^2 according to this picture is $(C_o - C'_-)x_D$.

If this is to be equivalent to the effect produced in the real double layer, then:

$$(C_o - C'_-)x_D = \int_{x=0}^{\infty} (C_o - C_-)dx, \quad (6)$$

where C' is given by equations (4) and (5) and C_- by Boltzman's equation (1).

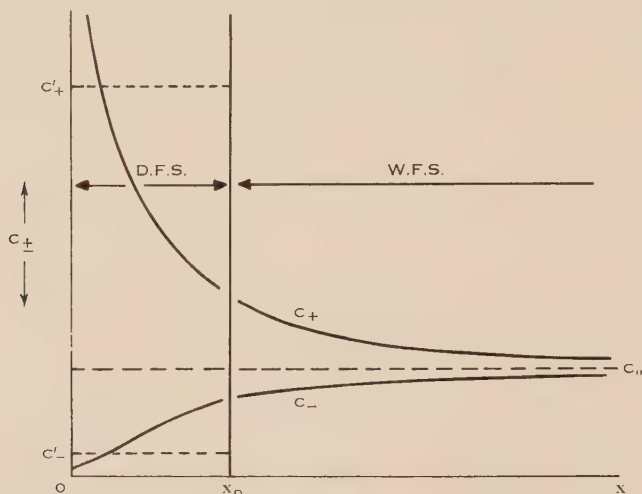


Fig. 2.—Diagrammatic representation of the extent of the Donnan free space (D.F.S.) and water free space (W.F.S.) near a negatively charged surface. C_{\pm} are the local concentrations of cation and anion at distances x from the surface. C_o is the concentration very far from the surface. C'_{\pm} are the equivalent concentrations in the D.F.S. which extends to x_D .

With the help of equations (1), (2), (4), and (5), equation (6) can be solved exactly for x_D , leading to the simple result:

$$x_D = \sqrt{(\epsilon RT / 2\pi F^2 C_o)}. \quad (7)$$

This is exactly twice the classical "width" of the electric double layer. Some values of x_D , which depends only on C_o and not on σ , are given in Table 1. For a bivalent electrolyte the electric double layer is relatively compressed, the value of x_D being smaller by a factor of $\sqrt{2}$ (Table 1). The average concentration of indiffusible anions in the double-layer system is given in line 4 of the table, calculated as $\sigma / (F \cdot x_D)$. This formula and theory apply only to a single, charged, plane surface in an infinite volume of solution. The theory should apply to a cylindrical surface provided the radius of the cylinder is more than twice or thrice x_D , but the problem becomes mathematically difficult in the presence of other charged surfaces, as this causes mutual disturbance of the two double layers. However, the theory is applicable if the surfaces are separated by at least $2x_D$.

In order to investigate the validity of the above approach it is necessary to have data on the apparent volume (V_D) of the D.F.S. and on the apparent concentration of indiffusible charge (A) in the D.F.S., as functions of the external concentration. The small amount of material available in the present experiment (single cell walls of *Chara australis*) precluded the separate and accurate determination of diffusible cation and anion in the material (see Briggs 1957), and an alternative attack was necessary. Fortunately, for our purpose it is only necessary to determine the ratio of divalent to monovalent cations in the material when it is equilibrated with definite external concentrations of a mixture of salts of specified divalent and monovalent ions.

TABLE 1
EXTENT (x_D) OF D.F.S. AND AVERAGE CONCENTRATIONS OF FIXED ANIONS AS
FUNCTIONS OF EXTERNAL ION CONCENTRATION

External ion concn. C_o (m-equiv/l)	0.1	1	10	100
x_D (uni-univalent) (Å)	608	192	60.8	19.2
x_D (bi-bivalent) (Å)	428	136	42.8	13.6
Av. concn. of fixed anions (m-equiv/l)*	17	54	170	540

* Calculated as $\sigma/(F \cdot x_D)$ for $\sigma = 10^{-5} \text{ C. cm}^{-2}$.

Although this approach requires extension of the double-layer theory to mixtures of ions of different valencies and there is then no simple expression for x_D , the value of x_D will lie between those values given in lines 2 and 3 of Table 1. The general argument, that because of the contraction of the double layer with increasing external salt concentration the apparent volume of the D.F.S. will decrease and the apparent A increase, is still correct.

Eriksson (1952) has shown that the ratio of bivalent ions to total counterions in a double-layer system containing sodium chloride and calcium sulphate (concentrations C_1 and C_2 equiv. cm^{-3} , respectively, in the bulk phase) is:

$$\text{Ca}_i/(\text{Ca}_i + \text{Na}_i) = 1 - \{C_1/2\sqrt{(z \cdot C_2)}\} \cdot \arg \sinh\{2\sqrt{(Z \cdot C_2)}/(C_1 + u_d \cdot C_2)\}, \quad (8)$$

where

$$Z = \pi\sigma^2/\epsilon RT,$$

and

$$u_d = 2 \cosh(-EF/RT),$$

(= 2 when the charged surfaces are infinitely separated).

If the cell wall phase of plant cells could be regarded as a homogeneous Donnan phase then, in terms of the mean concentration of indiffusible anions (A), the ratio

of calcium ions to total counterions is:

$$\text{Ca}_i/(\text{Ca}_i + \text{Na}_i) = 1 + C_1^2/C_2A - \sqrt{(C_1^4/4C_2^2A^2 + C_1^2/C_2A)} \quad (9)$$

(neglecting diffusible anions in the D.F.S.).

Comparison of observed successive replacements of calcium by sodium (Dainty, Hope, and Denby 1960) with the predictions of equations (8) and (9) may enable us to decide which system described best the cell wall phase.

III. EXPERIMENTAL AND RESULTS

Segments about 1 cm long of cell walls isolated from long internodal cells of *C. australis* were placed in a solution of CaCl_2 (1 m-equiv/l) for about 24 hr, and then treated for 2–3 days in the same solution with tracer amounts of ^{45}Ca , sufficient to give a specific activity of about $0.1 \mu\text{c}/\mu\text{-equiv}$. The segments were blotted and

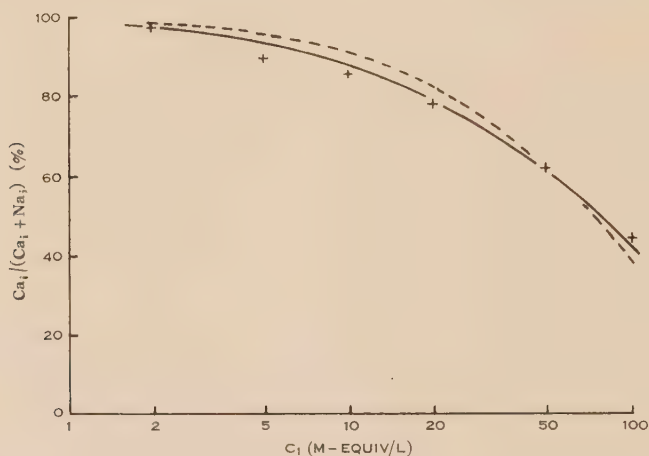


Fig. 3.—Variation in the exchangeable calcium (Ca_i) with external concentration of sodium or potassium (C_1) in segments of isolated cell walls from *Chara australis*, plotted as a fraction of the exchangeable calcium when sodium or potassium is absent ($\text{Ca}_i + \text{Na}_i$). Crosses are means of six determinations, half the height of the symbol being the standard error of the mean.

The full and dotted lines are theoretical relations explained in the text.

counted under a thin end-window G.M. tube. Individual segments were then returned to solutions all containing CaCl_2 at a concentration of 1 m-equiv/l and with NaCl or KCl added to give concentrations ranging from 2 to 100 m-equiv/l. After 7 days to reach equilibrium (see Dainty and Hope 1959) the segments were again blotted and counted. These determinations, together with measurements of specific activity of the solutions after the segments had reached equilibrium, enabled the ratio $\text{Ca}_i/(\text{Ca}_i + \text{Na}_i)$ to be calculated as a function of $[\text{Na}]_o$ or $[\text{K}]_o$, the denominator being taken as the amount of exchangeable calcium in the first treatment, and the numerator being taken as that in the second.

Little difference was found in the replacing power of sodium and potassium except possibly when their concentration reached 100 m-equiv/l. Figure 3 shows the results of such an experiment, six values for each concentration of sodium or potassium being combined for each point. The full line is the line of best fit using equation (8) and putting $\sigma = 4.2 \times 10^{-5} \text{ C. cm}^{-2}$. Nearly all the points of such experiments were bounded by curves for $\sigma = 3.3$ and 5×10^{-5} . The dotted line is the expected relation according to equation (9), with A put equal to 10 equiv/l.

The experimental results tend to be a better fit to the double-layer theory, all points except one being less than twice the standard error of the mean from the solid line. Other assumed values for A displace the dotted curve to left or right and do not give a better fit.

IV. DISCUSSION

The experimental results fit the expectations of the double-layer theory within reasonable limits. However, there are several assumptions involved:

- (1) Equation (7) is intended for a mixture of uni-univalent and bi-bivalent electrolytes, whereas the data were obtained using chlorides. This should make little difference, since only minute amounts of mobile anion are to be found in the D.F.S. in the wall at the concentrations used.
- (2) Ideal behaviour of ions is assumed throughout. It is well-known that the Gouy theory should be modified to account for ionic interaction, polarization of the ions, dielectric saturation, and finite ion size, which leads to a distance of closest approach between ions and the charged surface. Bolt (1955) points out that the differences between the corrected and simple theory largely cancel and the latter gives good agreement if the charge density does not exceed about $3 \times 10^{-5} \text{ C. cm}^{-2}$.
- (3) The curve in Figure 3 was calculated from equation (8) putting $u_d = 2$, i.e. assuming the charged surfaces are separated by distances such that the electric potential approaches zero at the point of symmetry. This is likely to be so more especially in higher concentrations of sodium chloride. At lower concentrations the effect of overlapping of the double layers is to raise the curve. For example, if $E = -100 \text{ mV}$ at the point of symmetry, then $u_d \simeq 54$ and the value at $[\text{Na}]_o = 2 \text{ m-equiv/l}$ is raised from 96.8 to 98.4%.

When the cell wall D.F.S. is described in terms of an assembly of Gouy double layers, the parameter "concentration of indiffusible anions" (A) is no longer appropriate. The apparent $A \{ = \sigma / (F \cdot x_D) \}$ in the present experiments varies between about 0.5 equiv/l ($[\text{Na}]_o = 2 \text{ m-equiv/l}$) and 2.3 ($[\text{Na}]_o = 100$), as x_D varies between 80 and 10 Å (cf. Dainty, Hope, and Denby 1960). The charge density is apparently constant at $4 \times 10^{-5} \text{ C. cm}^{-2}$.

When the double layers overlap enough, the A calculated from normal Donnan equations might not vary with external concentration. This may be the explanation of the results of Briggs, Hope, and Pitman (1958) who found the D.F.S. of disks

of red beetroot to have a constant volume of about 22 ml/kg fresh weight in concentrations of RbCl between 1 and 20 m-equiv/l. While this D.F.S. was ascribed to the cell cytoplasm, it has since (Pitman, personal communication) been located in the cell walls.

There are two sets of experimental data in the literature which illustrate the point we have tried to make in this paper that simple application of the Donnan equations to a charged colloidal system does not lead to values of D.F.S. and indiffusible charge concentration which are independent of external electrolyte concentration.

TABLE 2
VOLUME (V_D) AND EXTENT (x_D) OF D.F.S. IN GUM ARABIC SOLUTIONS AS FUNCTIONS
OF GUM ARABIC CONCENTRATION
Total volume of solution = 1000 cm³

Potassium Bromide Concn. (m-equiv/l)	Gum Arabic Concn. (m-equiv/l)	V_D (cm ³)	x_D (Å)	Av. Separation of Gum Arabic Particles (Å)
1.08	1	140	187	677
1.08	2	230	187	537
1.08	4	440	187	426
1.08	6	590	187	373
1.08	8	700	187	338
1.08	14	1000	187	281

Overbeek (1956) quotes some experimental results obtained by Klaarenbeek on the behaviour, as a Donnan system, of a colloidal solution of gum arabic, separated by a coarse membrane from a solution of potassium bromide. The concentration of bromide in the gum arabic solution was higher than expected from the assumption of a uniform Donnan system; in the phraseology of plant physiology the solution comprised a D.F.S. and a W.F.S. From Klaarenbeek's results we have calculated the volume of the D.F.S. (*a*), in Table 2, as a function of concentration of gum arabic solution at a constant potassium bromide concentration of 1.08 m-equiv/l; and (*b*), in Table 3, as a function of potassium bromide concentration at a constant gum arabic concentration of 1 m-equiv/l.

In the tables we also give the values of x_D (equation (7)), and the average separation of the gum arabic particles. The volume of the gum arabic solution is 1000 cm³.

It is clear from Table 2 that there is a rough proportionality between the volume of the D.F.S. and gum arabic concentration up to about 6 m-equiv/l when the average distance apart of the molecules is about $2x_D$. Thereafter the overlap of the double layers causes a marked departure from proportionality and when the gum arabic concentration is 14 m-equiv/l, the D.F.S. occupies the whole solution,

i.e. the solution is a uniform Donnan system. Note that this occurs when the average separation of gum arabic molecules is about $1.5x_D$, i.e. three times the double-layer thickness. This simple approach is vitiated to some extent by the Brownian motion of the molecules, but the mutual repulsion of the negatively charged particles tend to counteract this thermal motion and gives a lattice-like structure to the solution.

Table 3 shows how the volume of the D.F.S. markedly decreases as the potassium bromide concentration is increased from 1.08 to 100 m-equiv/l. The D.F.S. has in fact decreased more than it should according to the simple Gouy-Chapman theory (equation (7)); this may be due to experimental error which cannot be judged from Klaarenbeck's data, but it may be due to theoretical errors arising as a consequence of treating a sphere of radius 100 Å as a plane surface when the double layer has a thickness of up to 100 Å.

TABLE 3
VOLUME (V_D) AND EXTENT (x_D) OF D.F.S. IN GUM ARABIC SOLUTIONS AS FUNCTIONS OF
EXTERNAL POTASSIUM BROMIDE CONCENTRATION

Potassium Bromide Concn. (m-equiv/l)	Gum Arabic (Concn. (m-equiv/l))	V_D (cm ³)	x_D (Å)	Av. Separation of Gum Arabic Particles (Å)
1.08	1	115	187	677
10	1	25	61	677
100	1	6	19.4	677

This analysis of Klaarenbeck's data illustrates our point that the calculated D.F.S. does vary in the expected way with external concentration. It also shows that the simple theory is adequate provided that the neighbouring surfaces are more than $2x_D$ apart and that when the surfaces are closer together than $1.5x_D$, the whole system can be treated as if it were a uniform Donnan system.

The other set of results, to which reference has been made, is of more direct botanical interest. In a paper on "negative adsorption" of chloride ions, Schofield and Talibuddin (1948) express their results as the amount of water from which chloride is *absolutely* excluded per 100 g of dry jute fibre when equilibrated with various concentrations of potassium chloride. Here again, chloride is not excluded as much as it would be if the jute were a uniform Donnan system. From their results we calculate the data of Table 4. The total water volume is 75 cm³/100 g dry fibre and the total indiffusible charge is 22.0 m-equiv/100 g dry fibre.

From Table 4 it can again be seen that the volume of the D.F.S. in this plant material varies markedly with external solute concentration. Between $C_o = 1$ and 0.05 equiv/l the volume of the D.F.S. is inversely proportional to C_o but starts

to deviate from this at lower solute concentrations; since the extent of the D.F.S. is about 27 Å at $C_o = 0.05$, the onset of deviation at this point implies that the charged surfaces must be 50–60 Å apart. If the material has pores, they must have diameters of the same order of magnitude. The last column of the same table gives the estimated fixed anion concentration in the D.F.S. and this of course also varies as the volume of the D.F.S. varies.

TABLE 4

VOLUME (V_D) AND EXTENT (x_D) OF D.F.S. IN JUTE FIBRES AS FUNCTIONS OF EXTERNAL POTASSIUM CHLORIDE CONCENTRATION

Potassium Chloride Concn. (C_o) (m-equiv. cm ⁻³)	(V_D) (cm ³)	$V_D\sqrt{C_o}$	x_D (Å)	Apparent Indiffusible Ion Concn. (A) (equiv/l)
1.0	9.15	9.15	6.1	2.40
0.5	14.1	10.0	8.6	1.48
0.2	21.7	9.72	13.7	1.01
0.1	31.0	9.80	19.3	0.71
0.05	42.7	9.55	27.3	0.51
0.025	53.4	8.45	38.6	0.41
0.0125	61.5	6.88	54.6	0.36
0.00625	68.4	5.41	77.2	0.32
(0)	(75)			(0.29)

Since at low concentrations the volume of the D.F.S. is approaching the total water volume, it would seem that in jute fibre all the water is within about 100 Å of the fixed negative charges. Thus all the "pores" in jute fibres seem to be charged and have diameters in the range 50–200 Å. The situation seems to be quite different in the cell wall of *C. australis* (Dainty and Hope 1959), but it should be noted that "wet" jute fibre contains only 75 c.c./100 g dry weight whereas "wet" *Chara* cell wall contains 300 c.c./100 g dry weight.

V. ACKNOWLEDGMENTS

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DISTRIBUTION OF GROWTH AND ENZYME ACTIVITY IN THE DEVELOPING GRAIN OF WHEAT

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Summary

Ears of field-grown wheat were harvested on 12 occasions between anthesis and ripeness. Fresh weight, dry weight, and total nitrogen content of selected grains were determined.

On eight occasions similar grains were dissected into two future grain coat parts, designated "outer pericarp" and "rest of grain coat", and into endosperm and embryo. The resulting four fractions were analysed for total and for soluble protein content, for chlorophyll content, and for activity of glutamyl transferase, acid phosphatase, alanine-glutamic acid transaminase, and to a limited extent of alcohol dehydrogenase.

The results emphasize that the different parts of the grain successively commence their growth cycles and show distinct biochemical differentiation.

Glutamyl transferase activity is almost exclusively found in future grain coat tissues. A peak of activity, shown by the whole grain at day 12, can be accounted for by a peak of activity in the outer pericarp at that day. Specific activity is highest in this part of the grain.

Transaminase activity is very high in the endosperm even on a protein basis. The embryo shows the highest specific activity for alcohol dehydrogenase.

Acid phosphatase is found in all tissues but its specific activity is highest in those that show an early breakdown. In the outer pericarp acid phosphatase activity remains high after day 12 whilst glutamyl transferase activity and protein content drop.

The rest of the grain coat has similar enzyme activities to other parts but it is unique in its high chlorophyll content.

The mechanism establishing the distribution of glutamyl transferase is discussed.

I. INTRODUCTION

The implications of structure and biochemical differentiation for an understanding of the development of the wheat grain were brought to the fore in this Laboratory when it was found (Rijven and Banbury 1960) (i) that the enzyme glutamyl transferase showed a peak of activity early in the development of the grain; (ii) that this enzyme was almost exclusively localized in the maternal group of tissues; and (iii) that these tissues gradually degenerated after an early period of vigorous growth.

Dramatic changes occur in the grain between anthesis and ripeness. Such changes have been recognized in histological descriptions (Kudelka 1875; Percival 1921; Krauss 1933; Nutman 1939), but their significance has not yet been elucidated in physiological and biochemical terms.

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This paper presents a more detailed quantitative study of the development of the grain and its various parts in terms of protein content and enzyme activity. Thus not only growth was measured, but also biochemical differentiation.

II. MATERIAL AND METHODS

(a) *Plant Culture and Sampling*

In 1960, wheat (*Triticum sativum* L. cv. Festival) was grown in the field in rows 18 in. apart.

After emergence of the ears, those showing anthesis were tagged daily. On November 8 the day's yield of ears showing anthesis for the first time was 465. These ears were immediately retagged at random with labels of 12 different colours. This device enabled quick harvesting of a random sample on a particular harvest day. On such a day, 37 ears tagged with the same colour were collected. Harvests were made for the first seven times at 4-day intervals, then three times at 6-day intervals, and finally twice at 8-day intervals.

Out of each ear two grains were taken, viz. those of the first floret from two spikelets in the middle of the ear, i.e. one from each side of the ear. Thirty-six grains (one from each pair) were dissected and analysed as detailed below. Thirty of the remaining grains were used for determinations of fresh weight, dry weight, and total nitrogen content. The remaining grains were fixed in alcohol-formol-acetic acid for histological studies. The ears were stored deep-frozen.

(b) *Dissection and Designation of Parts*

Before and after dissection, the 36 grains were kept cool. Dissection was done for the first time on day 8 and was aimed throughout at separation of parts differing in cytological origin and metabolic function. For histological orientation see Plate 1.

(i) *The Maternal Group of Tissues*.—This group of tissues, i.e. the future grain coat, comprises the pericarp, integuments, and nucellus. It was divided into two fractions: "outer pericarp" and "rest of the grain coat".

The outer pericarp is parenchymatous and colourless, except near the groove where it contains some chlorophyll. Its separation is possible throughout due to early schizogeny of tissue bordering on the outside of the chlorophyll layer, and is achieved by a dismantling operation starting from the abaxial side of the grain. Near the groove, cutting with a fine scalpel is necessary to effect separation.

The rest of the grain coat is rather heterogeneous. Dissection of this fraction was started from the groove. Its main living part is the chlorophyll layer, which belongs to the inner layer of the pericarp. Another bulky component is the vascular bundle with adherent nucellus in the groove.

On the harvest of day 42 the rest of the grain coat and the endosperm were taken together.

(ii) *Endosperms*.—These could be easily isolated until day 16 inclusive, but the operation was continued until day 36 inclusive, although it became increasingly

more difficult, due to adhesion of the future aleurone layer to the membranous nucellus, which in fact had to be made part of the endosperm fraction. The number of endosperms and of grain coats sampled was reduced to 18 from day 24 onwards. This allowed replacement of any faulty dissections and made grinding, quantitative transfer, sampling, and extraction easier. Nevertheless, on the last two occasions, the endosperm and rest of the grain coat fractions may have been contaminated with each other. The results indicate that the general picture cannot have been seriously affected by this concession to practicability.

(iii) *Embryos*.—Embryos constituted a separate fraction for the first time on day 16, but were, at this stage, only a minute part (see Table 2).

(c) *Preparation of Extracts*

Each fraction, representing the equivalent of either 36 or 18 grains, was ground in a chilled mortar in the presence of 0.005M tris buffer [tris(hydroxymethyl)amino-methane], pH 7.4, then transferred quantitatively and made up to a volume of 9 ml. Samples to a total of 3 ml were withdrawn for determination of chlorophyll and total protein nitrogen content.

The extracts were then centrifuged for 10 min at 500 *g* and 1°C, the supernatants collected, and the residues resuspended in 6 ml of distilled water. The suspensions were again centrifuged. The combined supernatants were, if necessary, made up to 12 ml, each ml now representing 2 or 1 grain equivalents, and centrifuged for 60 min at 100,000 *g* and 1°C.

The supernatants, after this high-speed centrifugation, were used for several enzyme assays, and for protein nitrogen determinations. In the case of transaminase, two volumes of saturated ammonium sulphate were added to an aliquot of the supernatant. This was then centrifuged at 5000 *g* for 30 min and precipitates were redissolved in 0.10M phosphate buffer, pH 7.4.

(d) *Estimations and Enzyme Assays*

(i) *Fresh and Dry Weights*.—Fresh weights and dry weights (after drying overnight at 80°C) were determined on six replicates of five grains. For the harvest taken on the day of anthesis, however, these determinations were made on five replicates of 10 grains.

(ii) *Nitrogen Determinations*.—These were carried out on the dried grains and on the samples drawn from extracts. These samples were treated with trichloroacetic acid to a final concentration of 5% and the precipitates subjected to a microKjeldahl digestion procedure followed by nesslerization of the distillates, using Vanselow reagent. The estimates of samples, drawn before low- and after high-speed centrifugation, were taken to represent, respectively, total and soluble protein content. Whatever the exact interpretation of the latter fraction may be, it forms the logical basis for calculation of specific activities as the enzyme assays were made on these supernatants.

(iii) *Chlorophyll*.—Chlorophyll was estimated by adding 4 volumes of acetone to 1 volume of crude extract and, after centrifugation, reading the optical density at 652 m μ (Arnon 1949).

(iv) *Glutamyl Transferase*.—The procedure is essentially similar to the one used by Stumpf, Loomis, and Michelson (1951); 15-ml centrifuge tubes containing 0.1 ml 0.01M MnSO_4 , 0.1 ml 0.001M adenosine triphosphate, 0.1 ml 2M $\text{NH}_2\text{OH} \cdot \text{HCl}$ adjusted with NaOH to pH 6.5, 0.5 ml 0.2M tris-maleate buffer, pH 6.5, 0.2 ml 0.2M sodium arsenate, pH 6.5, 0.5 ml 0.5M glutamine extract (i.e. high-speed supernatant), and water to 2.25 ml were incubated at 30°C for 10 min and longer. The reaction was stopped by adding 0.75 ml of a mixture consisting of equal parts of 10% FeCl_3 in 0.1N HCl, 50% conc. HCl, and 24% trichloroacetic acid (Cowgill and Pardee 1957). After centrifugation, the optical density was measured at 540 m μ . Incubation mixtures without added glutamine were used as blanks. On no occasion did the optical density of these exceed a value of 0.020. Incubations, for periods

TABLE 1
WEIGHT AND TOTAL NITROGEN DATA OF DEVELOPING GRAIN OF WHEAT

Days after Anthesis	Fresh Weight (mg)	Dry Weight (mg)	Water Content (mg)	Solids (%)	Total Nitrogen (μg)
0	2.65	0.69	1.96	26.03	31.3
4	10.93	2.77	8.16	25.34	77.2
8	19.98	4.86	15.12	24.32	135.7
12	27.67	7.92	19.75	28.62	213.5
16	40.17	11.94	28.23	29.72	305.0
20	49.18	15.99	33.19	32.51	388.6
24	57.95	21.63	36.32	37.33	496.0
30	65.08	29.04	36.04	44.62	681.8
36	71.79	37.21	34.58	51.83	878.0
42	74.12	42.27	31.85	57.03	1126.6
50	65.83	45.40	20.43	68.96	1137.0
58	50.63	43.59	7.04	86.10	

up to 20 min, showed that the activity of extracts (O.D.'s <0.30) was linear with time, but with endosperm and embryo extracts, which had to be incubated for periods up to 1 hr, strict linearity was not always obtained because of very low activity (O.D.'s <0.10).

(v) *Acid Phosphatase*.—The assay followed a procedure described by Axelrod (1947). 1-ml aliquots of extract were incubated with 5 ml of 0.277M disodium *p*-nitrophenol phosphate in M/30 acetate buffer, pH 4.8, at 30°C. At regular time intervals 0.2-ml samples were withdrawn and added to 5 ml N/6 NaOH and the optical density measured at 400 m μ . Readings obtained from samples taken after 5 and 25 min of incubation were used to establish activity because it tended to decrease after longer periods of incubation.

(vi) *Transaminase*.—The assay followed a procedure described by Cohen (1955). Ammonium sulphate precipitates (66% saturation) redissolved in 0.1M phosphate buffer, pH 7.4, were used as the material to be assayed. 0.5-ml aliquots

of the purified extract were incubated at 30°C for several time intervals up to 1 hr with 100 μ moles alanine, 100 μ moles α -ketoglutarate, and with 0.1M phosphate buffer, pH 7.4, to a total volume of 2.2 ml. Pyridoxal phosphate was not added (Cruickshank and Isherwood 1958). The glutamic acid formed was determined manometrically using bacterial glutamic acid decarboxylase (Worthington Biochem. Corp., U.S.A.). On occasions, the results showed some unsystematic deviations from linearity with time, but the effect of error on the assessment of activity was reduced by taking all individual readings into account.

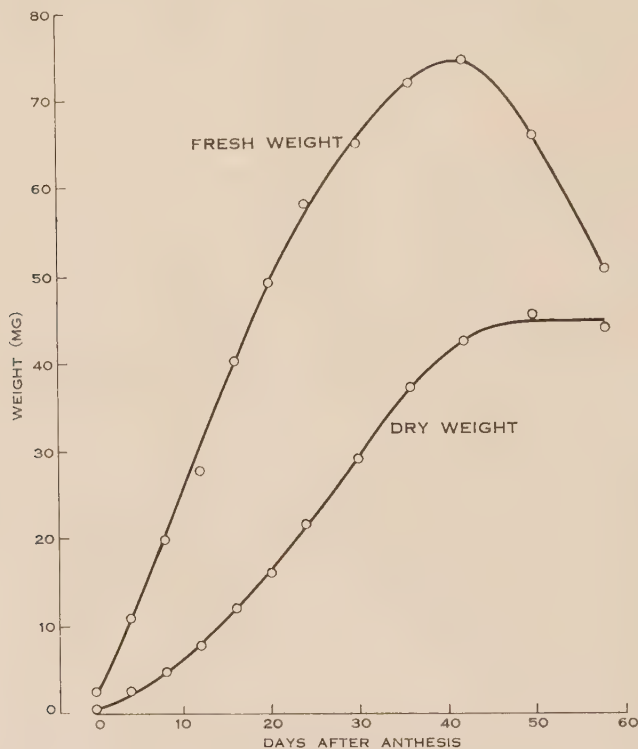


Fig. 1.—Fresh and dry weights of whole grain.

(vii) *Alcohol Dehydrogenase*.—The assay followed a procedure described by Racker (1955). Into a quartz cell (1 cm light path) was pipetted: 0.1 ml 0.06M diphosphopyridine nucleotide, 0.5 ml 0.1M sodium pyrophosphate, pH 8.5, 0.1 ml 3M ethanol, 2.2 ml water, and extract up to 0.1 ml. The optical density at 340 $m\mu$ was measured at 1-min intervals at a constant room temperature of 19°C.

III. RESULTS

(a) *Growth Pattern of Whole Grain*

Table 1 summarizes data on fresh weight, dry weight, and total nitrogen content of whole grains and includes derived data on water content and percentage solids. The weight data are plotted in Figure 1, in which it is seen that there were

no obvious deviations from the familiar sigmoid curve. This is noteworthy because the weather had shown considerable variation.

(b) *Distribution of Protein in Grain Parts*

For convenience of comparison, all primary data on grain parts have been accumulated into a single table (Table 2).

Direct information on grain parts was obtained for the first time on day 8. The main contributor to the total protein at this harvest is the outer pericarp (see also Fig. 2). There is no increase of protein in this fraction after this stage. However,

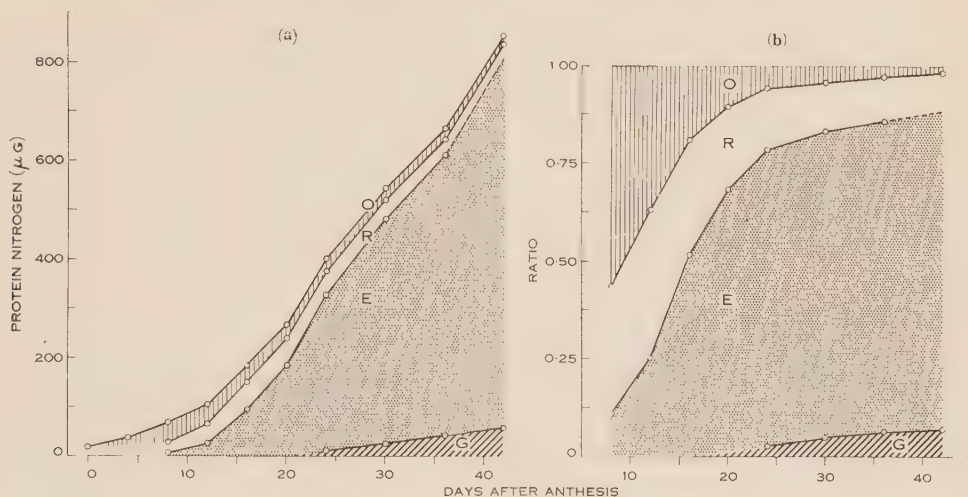


Fig. 2.—Distribution of protein nitrogen content in grain parts. (a) Additive plot in absolute values. (b) Additive plot in ratios of the whole grain. O, outer pericarp; R, "rest of coat"; E, endosperm; G, embryo (= germ).

it can be inferred from the data on the whole grain of day 0 and 4 that it must have started from a much lower value, and contributed then probably more to the total protein of the grain than all other parts taken together. A decrease in protein content, which continues, is noticed for the first time on day 16. The soluble protein fraction of the outer pericarp also follows this pattern.

A rise and fall in the total and soluble protein fraction is also shown by the rest of the grain coat. However, the increase continues until day 20 and higher absolute values are attained.

The importance of the maternal group of tissues during early development can be judged from the fact that until day 16 they contribute more than half of the total protein content of the grain (Fig. 2).

In contrast to these future grain coat tissues, the endosperm and embryo show a continuing increase in total protein content. The endosperm eventually contains close to 90% of that of the whole grain. It may be noted that less than 20% of the endosperm protein is soluble.

TABLE 2

DISTRIBUTION OF GROWTH AND ENZYME ACTIVITY IN THE DEVELOPING GRAIN OF WHEAT
 All data on a grain basis: W = whole grain, O = outer pericarp, R = "rest of coat", E = endosperm, G = germ

Days after Anthesis	Grain Part	Total Nitrogen (μg)	Total Protein Nitrogen (μg)	Soluble Protein Nitrogen (μg)	Chlorophyll Content (μg)	Glutamyl Trans-ferase ($\Delta\text{O.D./hr}$)	Acid Phos-phatase ($\Delta\text{O.D./hr}$)	Trans-aminase ($\mu\text{moles/hr}$)
0	W	31.3	19.9		0.15	0.18	0.036	
4	W	77.2	37.7	14.9		0.98	0.213	
8	W	135.7	69.8	28.2	2.09	3.18	0.338	
	O		39.3	15.1		2.715	0.264	
	R		22.8	9.6		0.450	0.069	
	E		7.7	3.5		0.014	0.005	
12	W	213.5	106.2	43.3	4.59	4.648	0.612	7.59
	O		39.3	16.5	0.38	3.750	0.350	0.39
	R		39.9	16.1	4.06	0.870	0.215	2.69
	E		27.0	10.7	0.15	0.028	0.047	4.51
16	W	305.0	183.9	71.3	7.08	3.192	0.956	37.40
	O		34.7	12.0		2.160	0.387	0.74
	R		53.7	20.1		0.990	0.356	4.41
	E		95.5	39.2		0.042	0.213	32.25
	G	1.6						
20	W	388.6	269.9	90.8		2.852	1.093	45.61
	O		28.3	11.8		1.605	0.398	0.32
	R		56.6	19.8		1.170	0.350	1.78
	E		185.0	59.2		0.077	0.345	43.51
	G	4.8						
24	W	496.0	401.9	118.4	7.08	2.020	1.161	48.11
	O		23.5	7.0		0.975	0.255	
	R		50.8	16.7		0.986	0.453	1.54
	E		316.6	89.8		0.059	0.453	46.34
	G		11.0	4.9				0.23
30	W	681.8	547.8	136.8	6.67	1.898	1.272	
	O		23.5	6.9		0.874	0.264	
	R		40.1	13.9		0.986	0.447	
	E		456.9	104.9		0.038	0.561	
	G		27.3	11.1				

TABLE 2 (*Continued*)

Days after Anthesis	Grain Part	Total Nitrogen (μg)	Total Protein Nitrogen (μg)	Soluble Protein Nitrogen (μg)	Chlorophyll Content (μg)	Glutamyl Trans-ferase ($\Delta\text{O.D./hr}$)	Acid Phos-phatase ($\Delta\text{O.D./hr}$)	Trans-aminase ($\mu\text{moles/hr}$)
36	W	878.0	668.0	152.8	4.52	1.042	1.121	69.89
	O		18.9	5.6		0.480	0.237	
	R		34.3	11.8		0.512	0.294	3.33
	E		572.6	116.1		0.032	0.516	66.56
	G		42.2	19.3		0.018	0.074	
42	W	1126.6	859.9	167.1	2.09	0.636	1.210	69.98
	O		15.7	4.0		0.291	0.155	
	R + E		786.0	140.0		0.320	0.945	67.56
	G		58.2	23.1		0.025	0.110	2.42

To illustrate the pattern of growth in the various tissues the total protein data of the different parts were recalculated as percentages of the maximum values attained by them, and are plotted in Figure 3. This diagram stresses the existence of a series of growth cycles, each characteristic for a particular grain part. This was first noticed by Nutman (1939) with respect to postfertilization phenomena in rye. Indeed it seems that the concept can be extended even beyond maturation because, during germination, the endosperm will also break down.

(c) *Distribution of Enzyme Activities in Grain Parts*

The enzymes assayed during a considerable period of the grain's development were: glutamyl transferase, acid phosphatase, and alanine-glutamic acid transaminase.

All direct information obtained on these enzymes is again given in Table 2. Absolute values on a grain basis are presented in a series of diagrams, paired with diagrams illustrating distribution ratios (Figures 4, 5, and 7).

Specific activities, using the soluble protein data as a basis, are given in Table 3.

(i) *Glutamyl Transferase*.—This investigation constitutes the third harvest series in which a peak of activity for the whole grain was found on the 12th day after anthesis. As stated previously (Rijven and Banbury 1960), this peak is not an artefact of extraction due to accumulation of inhibitors or of interfering enzymes. The peak is explained by the finding that the enzyme is almost exclusively localized in the future grain coat tissues, particularly in the outer pericarp. Although the activity decreases after day 12, concomitantly with protein loss, this tissue remains the most important contributor of glutamyl transferase activity (Fig. 4). As may be expected the specific activity of this tissue is considerably higher than of any other. It is also

TABLE 3

SPECIFIC ACTIVITIES OF ENZYMES IN PARTS OF DEVELOPING GRAIN OF WHEAT

Activities per mg soluble protein nitrogen: W = whole grain, O = outer pericarp, R = "rest of coat", E = endosperm, G = germ

Days after Anthesis	Grain Part	Glutamyl Transferase	Acid Phosphatase	Transaminase
4	W	65.77	14.29	
8	W	112.76	11.99	
	O	179.80	17.48	
	R	46.88	7.19	
	E	4.0	1.43	
12	W	107.34	14.13	175.29
	O	227.27	21.21	23.64
	R	54.04	13.35	167.08
	E	2.62	4.39	421.50
16	W	44.77	13.41	524.54
	O	180.0	32.25	61.67
	R	49.25	17.71	219.40
	E	1.07	5.43	822.70
20	W	31.41	12.04	502.31
	O	136.02	33.73	27.12
	R	59.09	17.68	89.89
	E	1.30	5.82	634.34
24	W	17.06	9.81	406.33
	O	139.29	36.43	
	R	59.04	27.13	92.22
	E	0.66	5.04	516.04
	G			46.94
30	W	13.87	9.30	
	O	126.67	38.26	
	R	70.91	32.16	
	E	0.36	5.5	
	G			
36	W	6.82	7.34	457.39
	O	85.71	42.32	
	R	43.39	24.92	282.20
	E	0.28	4.44	573.30
	G	0.93	3.83	
42	W	3.81	7.24	418.79
	O	72.75	38.75	
	R+E	2.29	6.75	482.57
	G	1.08	4.76	104.76

interesting to observe that whilst total and soluble protein remain constant in the outer pericarp between day 8 and 12, the activity of this enzyme increases there.

The relatively low activity of the endosperm fraction observed before (Rijven and Banbury 1960) now appears to apply throughout development. The activity could not be increased by dialysis.

(ii) *Acid Phosphatase*.—The distribution of this enzyme is much less specific, and to some extent reflects the distribution of protein (Fig. 5). However, it may be

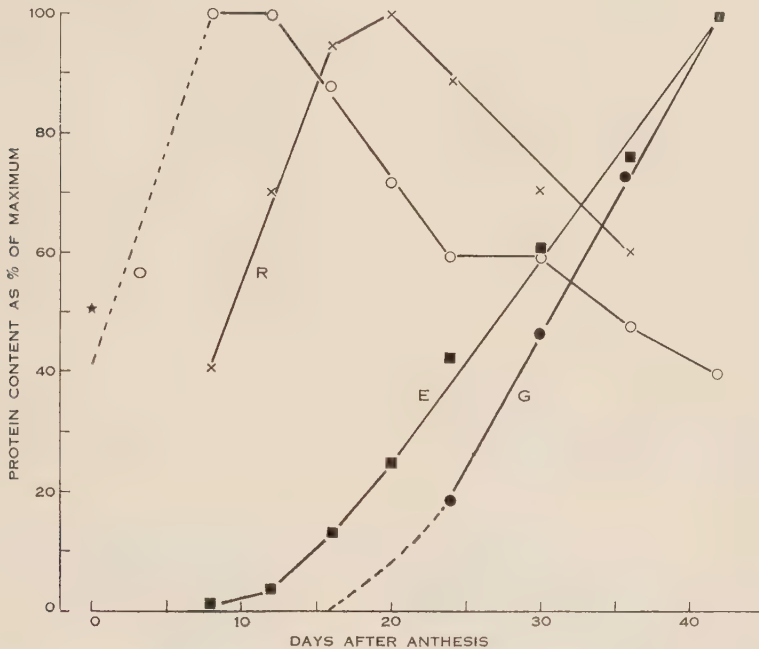


Fig. 3.—Protein nitrogen content of grain parts. Data are expressed as percentage of maximum value ever attained by the part. The asterisk at day 0 marks protein content of the whole grain expressed as a percentage of the maximum value attained by the outer pericarp. O, outer pericarp; R, "rest of coat"; E, endosperm; G, embryo (= germ).

noticed that the specific activity of the maternal tissues is significantly higher than that of the endosperm. This may well be connected with the early breakdown of these tissues. Another observation is that acid phosphatase activity remains high in the outer pericarp for some time after day 12 whereas protein content and glutamyl transferase activity fall off from then onwards. This is illustrated in Figure 6 by expressing the activities as percentages of the maximum attained. It is also apparent from this figure that throughout the period of physiological autolysis the relative activity of acid phosphatase is higher than that of glutamyl transferase. The possible significance of this pattern in relation to cellular enzyme localization will be considered in Section IV.

(iii) *Transaminase*.—Activity was found in all fractions, but the specific activity was highest in the endosperm. This, together with the fact that much more protein is accumulated there than anywhere else, makes the contribution of endosperm

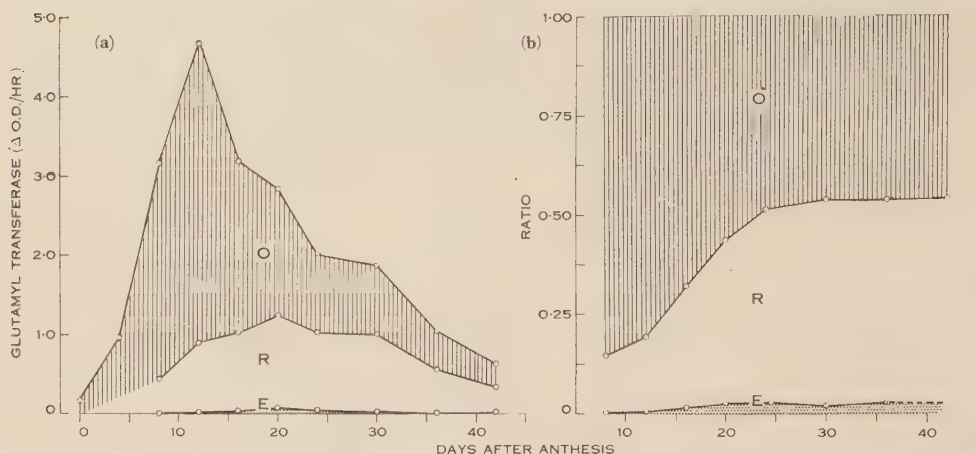


Fig. 4.—Distribution of glutamyl transferase activity in grain parts. (a) Additive plot in absolute values. (b) Additive plot in ratios of the whole grain. O, outer pericarp; R, "rest of coat"; E, endosperm; G, embryo (= germ).

to total activity high throughout (Fig. 7). The activity in the outer pericarp was low, in fact at the limit of detection possible by the methods employed. In the rest of the grain coat the specific activity was definitely higher than in the outer pericarp.

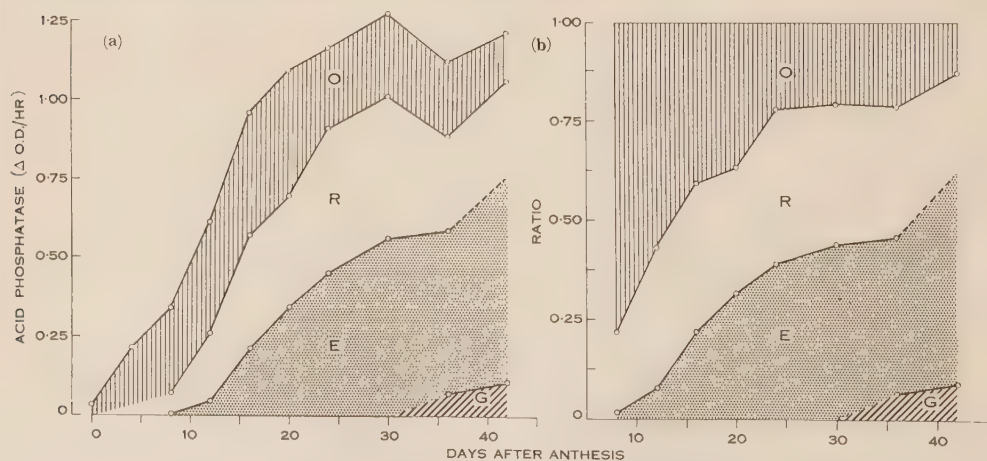


Fig. 5.—Distribution of acid phosphatase activity in grain parts. (a) Additive plot in absolute values. (b) Additive plot in ratios of the whole grain. O, outer pericarp; R, "rest of coat"; E, endosperm; G, embryo (= germ).

The rise of activity in the rest of the grain coat on day 36 (Table 2) is probably due to contamination by endosperm. The distribution of transaminase resembles that of leucine-activating enzyme (Rijven and Banbury 1960).

(iv) *Alcohol Dehydrogenase*.—This enzyme was only assayed in extracts of endosperms and of embryos of day 36. Endosperm showed higher activity on a grain basis ($\Delta O.D./hr = 21.84$ for endosperm, 12.60 for embryo), but on a soluble protein nitrogen basis the activity was higher in the embryo ($\Delta O.D./hr = 652.8$ per mg nitrogen for embryo, 188.1 per mg nitrogen for endosperm).

IV. DISCUSSION

Previous studies of changes in nitrogenous constituents in ripening seeds and cereal grains have been reviewed by McKee (1958). In general it is recognized that different proteins are synthesized at different stages and in particular that the reserve proteins are synthesized during the later stages of development. In the present

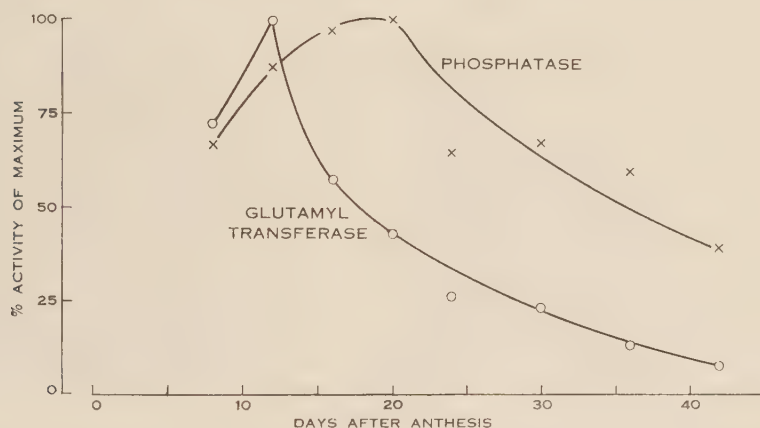


Fig. 6.—Glutamyl transferase and acid phosphatase activity in outer pericarp. Data expressed as percentage of maximum value attained therein.

investigation histological aspects of grain development have been taken into account and it is now established that the above feature, concerning the development of the whole grain, also holds for an aspect of protein synthesis in an important grain part, the endosperm. It can be readily established from Table 2 that up to day 16 soluble protein nitrogen contributes about 40% of the total protein nitrogen content of the endosperm; thereafter this percentage decreases until finally it is less than 20% of the total.

A peculiar fact revealed in these studies is the distribution pattern of glutamyl transferase. Comment has been made already on this fact in connection with glutamine synthesis in the developing grain and the possible metabolic role of the coat tissues (Rijven and Banbury 1960).

It may be emphasized now that the localization of this enzyme is not unique for wheat and that an essentially similar distribution has been found in barley and in a very different plant species, viz. pea (Rijven 1961*b*). In the developing pea seed the specific activity of the embryo is many times lower than that of the seed coat.

In attempting to explain this phenomenon, it is considered of importance to stress that in both cases the development of the maternal tissue parts precedes that

of the enclosed endosperm or embryo, and that the latter receive all their substrates from the maternal tissues. Further, attention must be drawn to observations which show that glutamyl transferase synthesis is repressed by glutamine. This has already been shown for HeLa cells by De Mars (1958). Using excised germinating wheat embryos it has now been established (Rijven 1961*a*) that exogenous glutamic acid stimulates synthesis of the enzyme to the same degree as protein synthesis. Glutamine, however, although stimulating protein synthesis, does not give a glutamyl transferase level beyond that of a control—no nitrogen—treatment, when it is applied in the range of 10–50 mM concentration. Addition of mixtures of glutamine and glutamic acid results in intermediate enzyme levels.

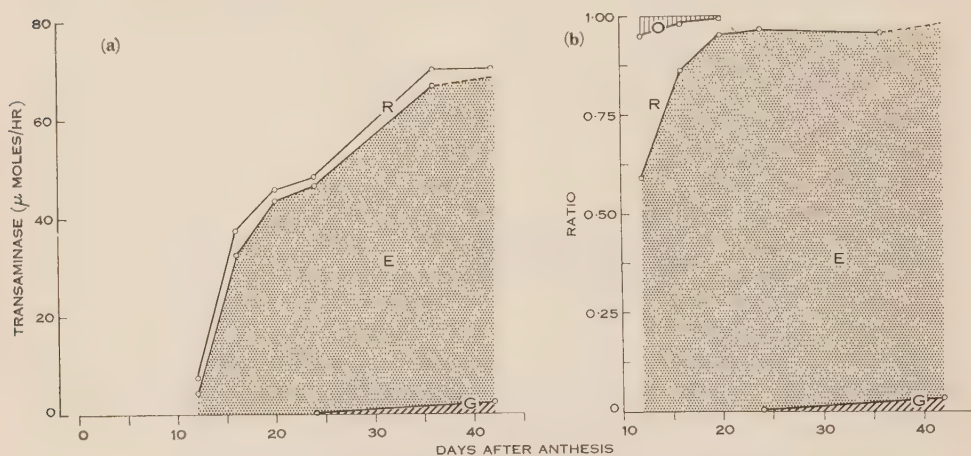


Fig. 7.—Distribution of alanine-glutamic acid transaminase in grain parts. (a) Additive plot in absolute values. (b) Additive plot in ratios of the whole grain. O, outer pericarp; R, "rest of coat"; E, endosperm; G, embryo (= germ).

These facts lead to the speculation that, in the grain or seed, a high glutamyl transferase level may become established in the earlier developing maternal parts because, in the incoming stream of nutrients, the amount of glutamic acid exceeds that of glutamine. Thus a "barrier" of glutamyl transferase may be established, preventing the passage of glutamic acid into the interior in amounts more than a fraction of the glutamine present, as during the course of its passage, glutamic acid would be converted into the amide by the enzyme. Hence in the interior, in endosperm or embryo, repression of the synthesis of the enzyme would follow. This hypothesis is not inconsistent with the observation that, upon germination the level of glutamyl transferase activity rises rapidly in both endosperm and embryo (Rijven 1961*a*). For in germinating barley, and presumably also in wheat, glutamic acid outweighs glutamine (labile amide) in the "soluble nitrogen" pool (Folkes and Yemm 1958). However, free glutamic acid is also present in the isolated developing endosperm at a concentration of at least 2.5 mM (Rijven, unpublished data). It would seem that to maintain the present explanation, compartmentalization of amino acid pools (Steward, Bidwell, and Yemm 1958) would be necessary.

Another observation of interest is that in the outer pericarp, acid phosphatase activity remains high after day 12, whilst glutamyl transferase activity and total protein content fall off (Fig. 6). Strikingly similar patterns of enzyme ontogeny have been observed during the autolysis of animal tissues, and their features have been embodied within the lysosome concept (de Duve 1958). There exist subcellular particles, lysosomes, containing a group of hydrolytic enzymes (amongst them acid phosphatase) which can break down cell constituents of major importance. However, the authors were unable to find evidence for the existence of such particles in young developing grains using osmotic shock or "Triton X100" treatment of extracts. An alternative solution to the localization and role of these enzymes is indicated by the finding of Lampion and Northcote (1960) that acid phosphatase activity of plant tissue culture material was largely bound up with a cell wall preparation suggesting it to be a surface enzyme.

With respect to the data on transaminase and alcohol dehydrogenase little comment seems necessary, but it may be pointed out that they provide evidence for biochemical differentiation; for any one of the parts studied it is now possible to mention an enzyme showing highest specific activity there. This does not apply strictly to the rest of the coat but its unique high chlorophyll content implies a whole complement of enzymes.

V. ACKNOWLEDGMENTS

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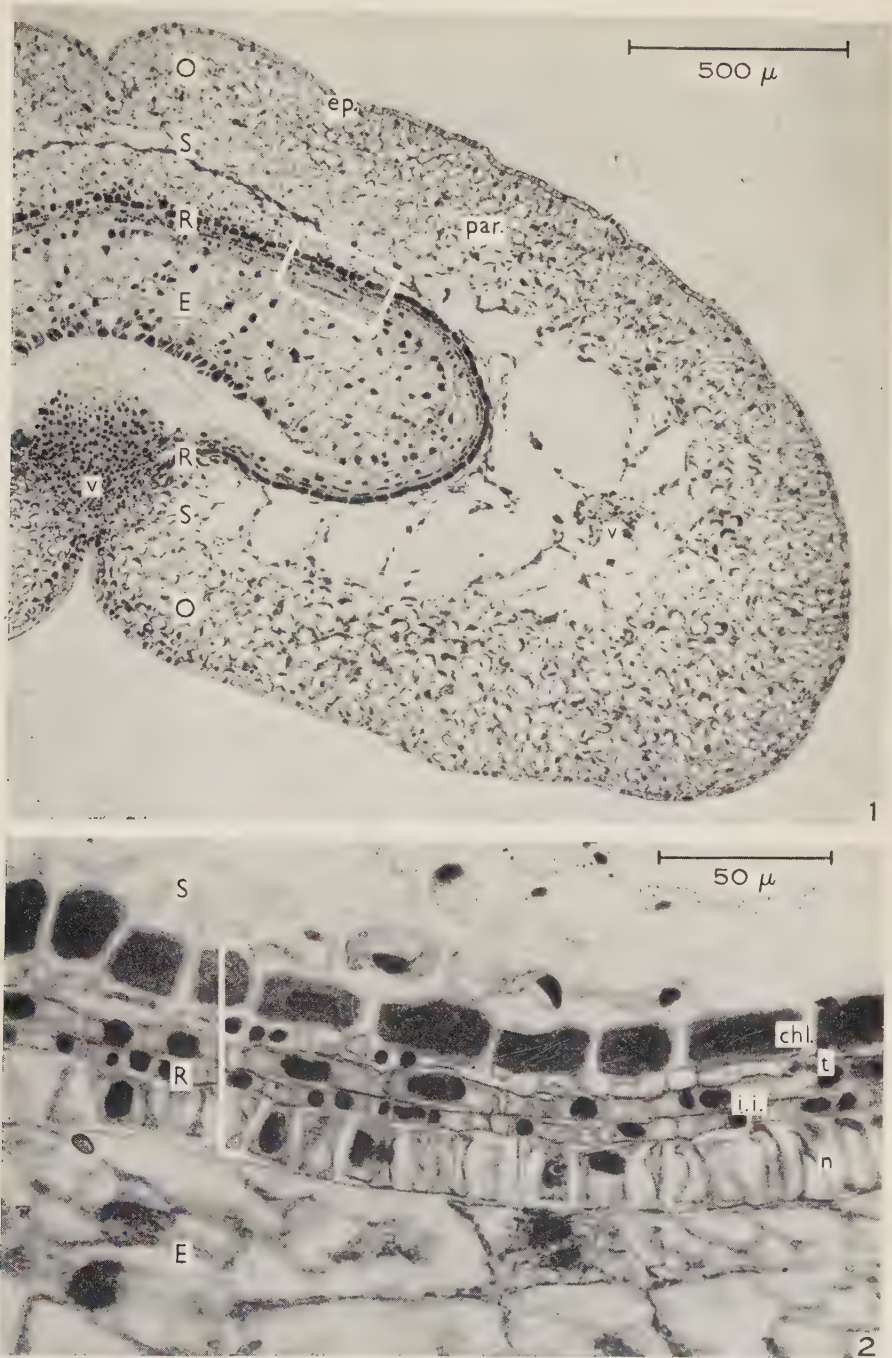
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EXPLANATION OF PLATE I

- Fig. 1.—Transverse section at low magnification through a grain of wheat harvested 12 days after anthesis. *O*, outer pericarp: *ep.*, epidermis on the outside; *par.*, parenchyma with starch granules; *S*, schizogenous gap separating outer pericarp from inner pericarp; *R*, rest of coat: *v*, vascular bundle. *E*, endosperm with large cells showing mitotic activity, but with no starch present as yet.
- Fig. 2.—Details at high magnification of a part similar to, but not identical with, the rectangle shown in Figure 1. *S*, *R*, and *E* as for Figure 1; *chl.*, chlorophyll layer—*in vivo* the cells of this layer are packed full with distinct chloroplasts at this stage; *t*, inner epidermis of pericarp—components are torn asunder, particularly on the lateral sides, and are known as "tubule cells"; *i.i.*, inner integument of two cell layers showing mitotic activity; *n*, epidermis of nucellus.

WHEAT GRAIN DEVELOPMENT



THE NITROGEN REQUIREMENTS OF SOME MEMBERS OF THE VIRIDANS GROUP OF STREPTOCOCCI

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Summary

The amino acid requirements of certain members of the viridans group of streptococci have been investigated. These requirements have not been found to be uniform among strains of *Streptococcus bovis*. Rumen strains of *Strep. bovis* require, in addition to 20 amino acids, rumen liquor or an extract of rumen liquor or certain other organic complexes. The chemical nature of the active factor suggests a peptide. The essential amino acids for the faecal strains of *Strep. bovis* were glutamic acid, aspartic acid, leucine, valine, asparagine, and histidine. The stimulatory amino acids were cystine, arginine, tryptophan, threonine, and lysine. *Streptococcus equinus* was shown to have similar nutritional requirements to *Strep. bovis*. The possibility that the species *Strep. equinus* should be discontinued is discussed. It was demonstrated with *Strep. equinus* that amino acid antagonism occurred between various combinations of amino acids and serine or glycine. The requirements of *Strep. salivarius* and related streptococci were found to be similar to each other. *Strep. salivarius* needed glutamic acid, cystine, alanine, and lysine, and was stimulated by aspartic acid or asparagine and by histidine or isoleucine or tyrosine. The related streptococci required cystine, alanine, and glutamic acid and were stimulated by lysine, leucine, aspartic acid or asparagine, and histidine. These organisms were considered varieties of *Strep. salivarius*. The transamination of oxaloacetic acid, pyruvic acid, and α -ketoglutarate is discussed.

I. INTRODUCTION

The viridans group of streptococci has been recognized since the last century but few investigations have been made on their nitrogen requirements. Previous workers have shown that the nitrogen requirements of *Streptococcus bovis* have varied from ammonium salts (with or without added CO₂) (Wolin, Manning, and Nelson 1959) to casamino acids (Wright 1960) through various numbers of amino acids (Niven, Washburn, and White 1948). *Strep. salivarius* has been shown to require seven amino acids (Smiley, Niven, and Sherman 1943) and no literature could be found concerning *Strep. equinus*. This paper reports on the nitrogen requirements of the section of the viridans group which does not grow at 50°C.

II. MATERIAL AND METHODS

The organisms used were all isolated by the author:

- (i) *Strep. bovis*, isolated from (a) sheep's rumen, (b) cow's intestine. Six rumen strains were studied. These had been obtained from sheep with rumen fistulae by plating with dilution into a medium containing 1% soluble

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starch, 0.5% "Oxoid" peptone, 0.3% "Oxoid" Lab-Lemco meat extract, and 1.5% agar at pH 6.8-7.0. Three intestinal strains were used. These had been isolated from cow's faeces by plating with dilution onto 1% glucose, 0.5% "Oxoid" yeast extract, 0.3% K_2HPO_4 , 1.5% agar, 0.02% sodium azide, and 0.5% bile salt.

TABLE 1
GROWTH OF A TYPICAL STRAIN OF STREP. BOVIS

Various fractions of rumen liquor were added to the control medium (20 amino acids plus the basal medium of Steele *et al.* (1949)). All fractions were the equivalent of 10% rumen liquor

Medium No.	Additions to Control Medium	Growth*	Medium No.	Additions to Control Medium	Growth*
1	Control	—	15	Residue of chloroform extraction	+++
2	Acid distillate of rumen liquor at pH 3	+	16	Alcohol extract, times 3	+++
3	Residue of acid distillate	++++	17	Residue of alcohol extraction	++
4	Alkaline distillate at pH 11	+	18	Alcohol-elutable material from charcoal adsorption	++++
5	Residue of alkaline distillate	++++	19	Ethyl acetate-elutable material from charcoal adsorption	+
6	Dialysable material	++++	20	Solution of ash	—
7	Non-dialysable material	+++	21	Acid hydrolysable material (5N HCl for 18 hr)	—
8	Ether extract, times 5	+++	22	Alkaline fraction of ionophoresis of Synge (1951)	++
9	Residue of ether extraction	++++	23	Neutral fraction	++++
10	Non-charcoal adsorbable residue	—	24	1% acetic acid fraction	++++
11	Rumen liquor boiled (sealed) for 1 hr	++++	25	0.1N sulphuric acid fraction	++
12	Butanol extract, times 2	+++	26	10% rumen liquor	++++
13	Residue of butanol extraction	+++			
14	Chloroform extract, times 3	++			

* The approximate relationship between visual and turbidimetric readings on a Unicam SP450 spectrophotometer were:

Visual	Optical Density	Visual	Optical Density
+	0.10	+++	0.30
++	0.20	++++	0.50

- (ii) *Strep. salivarius*, isolated from the human mouth. Two strains were isolated by plating throat swabbings onto 5% sucrose, 1.0% "Difco" tryptone, 0.5% "Oxoid" yeast extract, 0.3% K_2HPO_4 , 0.02% sodium azide, and 1.5% agar, at pH 7.0, and selecting mucoid, dome-shaped colonies.

- (iii) Streptococci closely related to *Strep. salivarius*, isolated from the human mouth. Two strains were studied after isolation by the same method as for *Strep. salivarius*.
- (iv) *Strep. equinus*, isolated from the horse's intestine. One strain was studied. This was isolated by the same method as intestinal *Strep. bovis*.

All organisms except (iii) agreed with the description given in "Bergey's Manual" (Breed, Murray, and Smith 1957). These organisms (iii) differed from *Strep. salivarius* in that they did not ferment inulin or produce mucoid colonies on sucrose agar.

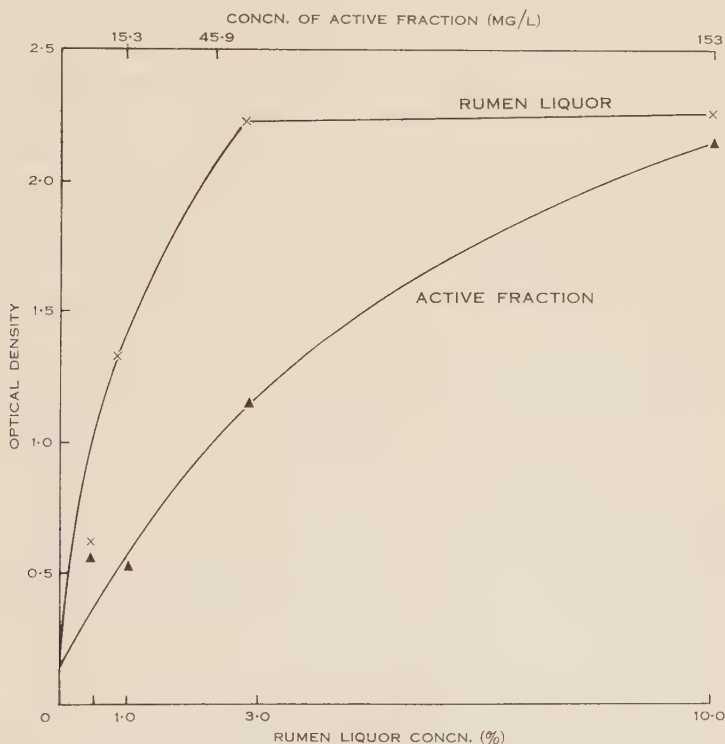


Fig. 1.—The growth curve of a typical rumen strain of *Strep. bovis* with increasing concentration of rumen liquor or active fraction of rumen liquor added to all amino acids and other nutrients.

All chemicals used were A.R. grade where possible.

Measurements of optical density were made visually or photometrically. The spectrophotometers used were Bausch and Lomb, Spectronic 20 (for rumen strains) and Unicam SP450 (for remaining organisms).

III. PROCEDURE

The bacterial cells were grown in flasks containing 1% glucose, 1% "Oxoid" peptone, and 0.5% "Oxoid" bacteriological yeast extract for 24 hr at 37°C. The cells were centrifuged, suspended in phosphate solution at pH 7.0, recentrifuged, and

resuspended in phosphate solution at pH 7.0. They were then inoculated into various media and incubated at 37°C for 24 hr. The media for rumen organisms was sterilized by Seitz filtration and for other organisms by autoclaving at 10 lb for 10 min as recommended by Steele *et al.* (1949).

The carbohydrates, vitamins, purines, and salts were chemically identical with those used by Steele *et al.* (1949). To these were added amino acids in the proportions given by these authors.

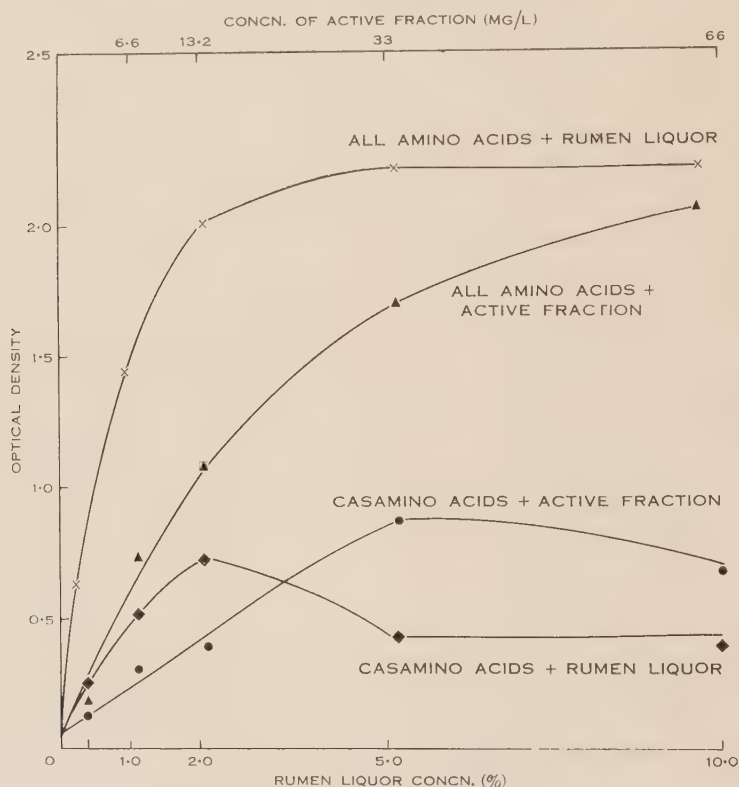


Fig. 2.—The growth curve of a typical rumen strain of *Strep. bovis* in (a) all amino acids with either rumen liquor or active fraction, or (b) casamino acids with either rumen liquor or active fraction.

IV. RESULTS

(a) Rumen Strains of *Strep. bovis*

These organisms did not grow with the addition of 20 amino acids to the basal medium of Steele *et al.* (1949). Growth would proceed if various organic complexes were added to the basal medium plus 20 amino acids. These included peptone, tryptone, meat extract, yeast extract, and strained rumen liquor. Growth was shown to be proportional to concentration of peptone or rumen liquor (see Fig. 1).

The rumen liquor was further investigated. Representative results are shown in Table 1 of growth of a typical strain of *Strep. bovis* in various fractions of rumen

liquor, which were added to the basal medium plus 20 amino acids. All fractions were the equivalent of 10% rumen liquor.

An extract showing activity was prepared by shaking 100 g charcoal with 500 ml highly centrifuged rumen liquor for 2 hr. This was filtered, washed, and eluted with 500 ml 50% alcohol, with shaking for 1 hr. The charcoal was washed with alcohol. The material was recovered by vacuum distillation.

This fraction showing the activity was added in various amounts to the basal medium. This was repeated many times and it was found that growth was proportional to this additive to the limits of the investigation (equivalent to 10% rumen liquor). The resultant growth was slightly less than with rumen liquor (see Fig. 1).

Total nitrogen was estimated on this extract by the microKjeldahl method as 4.2%. Nitrogen in the form of ammonia was estimated by the Conway method and was 1.1%. Thus the non-ammoniacal nitrogen was 3.1%.

A solution of this extract showing activity was prepared containing 0.66 mg/ml and subjected to ultraviolet spectrophotometry from 220 to 350 m μ . The results showed no peaks indicating that the material did not contain any aromatic rings.

Vitamin-free casein hydrolysate was substituted for the sum of individual amino acids and this was found to be antagonistic to the rumen liquor or the active fraction of rumen liquor (see Fig. 2).

When rumen liquor was used the simpler medium of Niven, Washburn, and White (1948) could not be substituted for the mixture of all amino acids and vitamins of Steele *et al.* (1949), showing that rumen liquor alone was not sufficient for the organisms.

(b) Faecal Strains of *Strep. bovis*

The minimum number of amino acids which would support visible growth of these strains was investigated. To these were added the remaining amino acids to determine the number which would give growth equal to that with all amino acids. The results are set out in the following tabulations:

Medium No.	Amino Acids Used	Result	Comments
A(1)	Glutamic acid, leucine, arginine, methionine, lysine, tyrosine, isoleucine, histidine, tryptophan, alanine, asparagine, aspartic acid, cystine	No growth	
A(2)	Medium A(1) + glutamine, glycine, threonine, valine	Growth	One or more amino acids of this group was necessary for <i>Strep. bovis</i>
A(3)	Medium A(1) + valine	Growth	Remaining three did not give a response
A(4)	Glutamic acid, leucine, arginine, methionine, lysine, tyrosine, isoleucine, histidine, phenylalanine, proline, serine, tryptophan, valine	No growth	One or more of the group aspartic acid, cystine, alanine, and asparagine was required

A(5)	Medium A(4) + asparagine + aspartic acid	Growth	No growth with addition singly or other pairs
A(6)	Glutamic acid, leucine, arginine, methionine, lysine, tyrosine, isoleucine, histidine, aspartic acid, asparagine, valine	Growth	No requirement for phenylalanine, proline, serine, tryptophan
A(7)	Glutamic acid, leucine, arginine, methionine, aspartic acid, asparagine, valine	No growth	One or more of lysine, tyrosine, isoleucine, and histidine required
A(8)	Medium A(7) + histidine	Growth	Histidine was essential, no response to any of the other amino acids
A(9)	Valine, aspartic acid, asparagine, histidine	No growth	One or more of glutamic acid, arginine, or leucine required
A(10)	Medium A(9) + glutamic acid + leucine	Growth	Methionine and arginine had no effect on growth

The minimum number of amino acids supporting growth was valine, aspartic acid, asparagine, histidine, glutamic acid, and leucine. Growth with all amino acids was greater and therefore the nature of the stimulation was investigated:

Medium No.	Amino Acids Used	Result	Comment
B(1)	Glutamic acid, leucine, valine, asparagine, aspartic acid, histidine, + single addition of each remaining amino acid	Stimulation with cystine	Less growth than with all amino acids
B(2)	Medium B(1) + cystine + single addition of each remaining amino acid	Stimulation with arginine	Less growth than with all amino acids but greater than with medium B(1) + cystine
B(3)	Medium B(1) + cystine + arginine + single addition of each remaining amino acid	Stimulation with tryptophan, threonine, or lysine	Less growth than with all amino acids but greater than with medium B(1) + cystine + arginine. Tryptophan slightly more effective than either of other amino acids
B(4)	Medium B(1) + cystine + arginine + tryptophan + single addition of each remaining amino acid	Stimulation with threonine	Less growth than with all amino acids but greater than with medium B(1) + cystine + arginine + tryptophan
B(5)	Medium B(1) + cystine + arginine + tryptophan + threonine + single addition of each remaining amino acid	Stimulation with lysine	Equal to growth with all amino acids, greater than with medium B(1) + cystine + arginine + tryptophan + threonine

The stimulatory amino acids were shown to be cystine, arginine, tryptophan, threonine, and lysine in the presence of each other.

(c) *Strep. equinus* from the Horse's Intestine

The amino acid requirements of this organism were investigated with the same method as that used for *Strep. bovis*:

Amino Acids Used	Result	Comment
1. Medium A(1)	No growth	
2. Medium A(2)	Growth	One or more of these amino acids required
3. Medium A(1) + valine	Growth	No response from other three
4. Medium A(4)	Some growth with cystine or asparagine	Less than with aspartic acid, cystine, alanine, and asparagine together
5. Medium A(4) + asparagine + aspartic acid or cystine	Growth	No other combinations were effective
6. Medium A(6)	Growth	No requirement for phenylalanine, proline, serine, tryptophan
7. Medium A(7)	Growth	Less than with medium A(7) + lysine, tyrosine, isoleucine, histidine
8. Medium A(7) + histidine	Growth	Equal to that with all four amino acids present. Histidine was stimulatory
9. Medium A(9)	Not tested	

The essential amino acids were the same as for *Strep. bovis* strains from the cow's intestine as far as they were examined. The nature of the stimulation was investigated, as for *Strep. bovis*.

Amino Acids Used	Result	Comment
1. Medium B(1)	Stimulation with cystine which was greater than for <i>Strep. bovis</i>	Less growth than with all amino acids
2. Medium B(2)	Stimulation with arginine, less than for <i>Strep. bovis</i>	Growth with medium B(1) + cystine + arginine better than with <i>Strep. bovis</i>
3. Medium B(3)	Slight stimulation with tryptophan, threonine, or lysine. Less than for <i>Strep. bovis</i>	Growth with medium B(1) + cystine + arginine + tryptophan equal to that with <i>Strep. bovis</i>
4. Medium B(4)	Stimulation with threonine, equal to that for <i>Strep. bovis</i>	Growth equal to that of <i>Strep. bovis</i>

5. Medium B(5)	Stimulation with lysine. Same degree of stimulation as for <i>Strep. bovis</i>	Growth equivalent to that of <i>Strep. bovis</i>
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The resultant requirements were the same as for *Strep. bovis*, but the degree of stimulation of the various combinations was slightly different from that for *Strep. bovis*. The addition of glycine or serine to every combination of amino acids inhibited growth.

(d) *Oral Strains of Strep. salivarius and Related Streptococci*

Medium No.	Amino Acids Present	Result	Comment
(1)	Glutamic acid, leucine, arginine, methionine, lysine, tyrosine, isoleucine, histidine, phenyl-alanine, proline, serine, tryptophan, alanine, asparagine, aspartic acid, cystine, glutamine, glycine, threonine, valine	Good growth of <i>Strep. salivarius</i> and related streptococci	
(2)	Glutamic acid, leucine, arginine, methionine, lysine, tyrosine, isoleucine, histidine, phenyl-alanine, proline, serine, tryptophan, alanine, asparagine, aspartic acid, cystine	Good growth of <i>Strep. salivarius</i> and related streptococci	Glutamine, glycine, threonine, valine not required
(3)	Glutamic acid, leucine, arginine, methionine, lysine, tyrosine, isoleucine, histidine, phenyl-alanine, proline, serine, tryptophan	No growth of either group of organisms	At least one of cystine, alanine, aspartic acid, and asparagine required
(4)	Medium (3) + cystine	Slight growth of <i>Strep. salivarius</i> . No growth of related streptococci	No response to alanine, aspartic acid, or asparagine or combinations of these
(5)	Medium (3) + cystine + alanine	Good growth of both types of organisms	No growth if alanine present without cystine. Both require cystine + alanine
(6)	Medium (3) + cystine + alanine + aspartic acid or asparagine	Good growth of both types of organisms. Better than (5)	All require cystine + alanine + aspartic acid or asparagine
(7)	Glutamic acid, leucine, arginine, methionine, lysine, tyrosine, isoleucine, histidine, cystine, alanine, aspartic acid, or asparagine	Good growth of both groups of organisms	No requirement for tryptophan, serine, phenylalanine, proline

(8)	Glutamic acid, leucine, arginine, methionine, cystine, alanine, aspartic acid, or asparagine	No growth of <i>Strep. salivarius</i> Growth of related streptococci [less than (7)]	<i>Strep. salivarius</i> required one or more of lysine, tyrosine, isoleucine, histidine. Related streptococci need one or more for stimulation
(9)	Medium (8) + lysine	Growth of <i>Strep. salivarius</i> [less than (7)]. Good growth of related streptococci [equal to (7)]	Lysine was essential for <i>Strep. salivarius</i> and stimulatory for related streptococci. Tyrosine, isoleucine, histidine had no effect on <i>Strep. salivarius</i> ; slight stimulation of related streptococci
(10)	Medium (8) + lysine with tyrosine, isoleucine, or histidine	Good growth of <i>Strep. salivarius</i> [equal to (7)]. Good growth of related streptococci, some stimulation with lysine + histidine	Histidine, isoleucine and tyrosine stimulate <i>Strep. salivarius</i> in the presence of lysine. Some additional stimulation of related streptococci on addition of histidine to lysine
(11)	Lysine, cystine, alanine, aspartic acid or asparagine, histidine	No growth of either group of organisms	At least one of glutamic acid, arginine, methionine, or leucine required
(12)	Medium (11) + glutamic acid	Good growth of both groups of organisms	Glutamic acid is essential. No effect with methionine, leucine, or arginine
(13)	Medium (11) + glutamic acid + leucine	Good growth of <i>Strep. salivarius</i> . Better growth of related streptococci than in (12)	Leucine in the presence of glutamic acid was stimulating for the related streptococci

The organisms *Strep. salivarius* and the related streptococci were tested with keto acids in place of certain amino acids. These were α -ketoglutaric acid for glutamic acid, sodium pyruvate for alanine, and oxaloacetic acid for asparagine.

One strain of *Strep. salivarius* could use α -ketoglutarate, but not the other keto acids, whereas the other strain could use none of these keto acids. The related streptococci could use pyruvate but neither of the other keto acids.

V. DISCUSSION

(a) *Strep. bovis*

Strains of *Strep. bovis* of different origin, namely isolates from sheep's rumen and cow's intestine, were shown to have different nutritional requirements.

In addition to all the amino acids, the rumen strains needed complex organic material, the most effective of which was rumen liquor. Rumen liquor contains much

nitrogen and many growth factors, and was the initial source of the organisms. McNeil, Doetsch, and Shaw (1954) found that no other material was as stimulatory for the overall rumen population as rumen liquor.

Peptone also supported good growth of these organisms. Habeeb (1957) showed that peptone contained about 50 different peptides in addition to free amino acids. Diaminopimelic acid could not substitute for any organic complexes.

The growth with active fraction of rumen liquor was important, and it was probably due to a peptide or peptides. Other workers have reported that the basis of stimulation of growth of organisms by complex materials is due to degradation products of ribonucleic acid or by peptides from enzymatic digestion of proteins (Ikawa and O'Barr 1956). In this case no nucleoside was involved, as no ring structure could be shown to be present. The chemical properties were indicative of a peptide, i.e. presence of non-ammoniacal nitrogen and loss of activity on acid hydrolysis. The requirement for a peptide as a growth factor is not unusual in the family Lactobacillaceae. Guss and Delwicke (1954) found that *Strep. thermophilus* could not use an acid digest of casein, but required in addition the residual peptides of tryptic digest of casein. For other examples, see Slade, Knox, and Slamp (1951) (*Strep. pyogenes*), Mergenhagen and Scherp (1957) (*Strep. putridus*), Wright and Skeggs (1944) (*Strep. lactis*, *Strep. faecalis*), Kitay and Snell (1950) (lactobacilli), Peters, Prescott, and Snell (1953) (*Lactobacillus delbrueckii*), Kihara, Klatt, and Snell (1952) (*L. casei*), Woolley and Merrifield (1958) (*L. casei* and some streptococci).

It was interesting that the complete range of amino acids could not be exchanged for casamino acids. This was possibly due to antagonistic peptides (Peters and Snell 1954; Woolley and Merrifield 1958).

The nitrogenous needs of the faecal strains of *Strep. bovis* could be met by amino acids. The nitrogenous requirements of *Strep. bovis* are not uniform within the species. This was commented upon by Hutner (1938) and it has been amplified by other workers. Wolin, Manning, and Nelson (1959) found that some growth occurred without CO₂ in the presence of ammonium salts, whereas Wright (1960) describes a requirement for casamino acids. Niven, Washburn, and White (1948) found that two amino acids were satisfactory, whereas for two of 14 strains of *Strep. bovis* used by Smiley, Niven, and Sherman (1943) seven amino acids were sufficient and an additional strain required casein hydrolysate.

This phenomenon of lack of nutritional uniformity within the species is not confined to *Strep. bovis*, but exists with other streptococci, cf. Pray (1942) and Guss and Delwicke (1954) for *Strep. thermophilus*; Shuman and Farrell (1941), Niven and Sherman (1944), and McCoy and Wender (1953) for *Strep. faecalis*; Anderson and Elliker (1953) and Niven (1944) for *Strep. lactis*.

(b) *Strep. equinus*

Strep. equinus had very similar nutritional requirements to *Strep. bovis* isolated from cow's intestine. This leads one to consider the observations of Seeley and Dain (1960), who feel that the validity of maintaining two species must be reconsidered. These workers feel that the differences between the species as given in Bergey (1957) are very small and that the isolation from horse faeces of *Strep.*

bovis by Higginbottom and Wheeler (1954) further weakens the taxonomic position. Therefore the possibility that *Strep. equinus* is a non-lactose-fermenting variant of *Strep. bovis* must be considered.

The author could find no papers on the nitrogen requirements of an organism fitting the description of *Strep. equinus*.

Inhibition in the presence of serine or glycine has been reported by other workers. In this experiment glutamic acid and valine were always present with serine or glycine. This agrees with McCoy *et al.* (1954). O'Barr, Levin, and Reynolds (1958) reported antagonism between various combinations of serine, glycine, threonine, and alanine. In the present investigation inhibition occurred without alanine and with or without threonine. It may be concluded that serine and glycine inhibited the growth of *Strep. equinus*, but not of *Strep. bovis* under similar conditions, and this represents the main nutritional difference between the species. This type of inhibition is known amongst bacteria and probably represents competition for an enzyme involved in a certain metabolic pathway.

(c) *Strep. salivarius*

Strep. salivarius was shown to have different requirements from those reported by Smiley, Niven, and Sherman (1943). However, the medium reported by these authors would only support growth in two-thirds of their strains.

The relationship between *Strep. salivarius* and other streptococci of this group is quite close. The major difference was the use of lysine. It is difficult to attempt classification, but these organisms seem closely related to *Strep. salivarius*, and it is uncertain whether the different fermentation and cultural characteristics alone would warrant the establishment of a different species.

The substitution of keto acids for amino acids revealed a difference between the two strains of *Strep. salivarius* and a uniformity between the related streptococci. The results disagreed with those of Wright (1960), who found that the related organism, *Strep. bovis*, could transaminate oxaloacetic acid.

The important findings of this investigation are:

- (1) The lack of uniformity of nitrogen requirements between members of the species *Strep. bovis*.
- (2) The rumen strains of *Strep. bovis* require complex organic material as well as all amino acids, vitamins, and purines for growth. The active material was probably a peptide.
- (3) The faecal strains of *Strep. bovis* required glutamic acid, aspartic acid, leucine, valine, asparagine, and histidine, and were stimulated by cystine, arginine, tryptophan, threonine, and lysine in the presence of each other.
- (4) The amino acid requirements of *Strep. equinus* were similar to those of the faecal strains of *Strep. bovis*, and it is thought that this organism may be a variant of *Strep. bovis*, rather than a separate species.
- (5) With *Strep. equinus* the addition of glycine or serine to various mixtures of amino acids resulted in inhibition, thus demonstrating amino acid antagonism.

- (6) The amino acids glutamic acid, cystine, alanine, and lysine were found to be essential requirements for *Strep. salivarius*, and aspartic acid or asparagine, and histidine or isoleucine or tyrosine were stimulatory.
- (7) The related streptococci had similar requirements. The essential amino acids were cystine, alanine, and glutamic acid and the stimulatory ones were lysine, leucine, aspartic acid or asparagine, and histidine. As these two organisms were shown to have similar nutritional requirements, the related streptococci were probably variants of *Strep. salivarius*.
- (8) None of the related streptococci could transaminate oxaloacetic acid, *a*-ketoglutarate, or pyruvic acid, whereas one strain of *Strep. salivarius* could transaminate pyruvate and the other could transaminate *a*-ketoglutarate.
- (9) Both *Strep. bovis* and *Strep. salivarius* require many amino acids. *Strep. bovis* needs 11, and *Strep. salivarius* needs six amino acids for good growth. This type of similarity would be expected from members of closely related genera.

VI. ACKNOWLEDGMENTS

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THE INFLUENCE OF TESTOSTERONE TREATMENT ON THE DEVELOPMENT OF THE BURSA OF FABRICIUS IN THE CHICK EMBRYO

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Summary

1. The histological development of the bursa of Fabricius in the chicken is described.

2. By early administration of testosterone to the embryo the development of the bursa is completely inhibited.

3. At a later stage testosterone results in a more or less complete replacement of lymphoid follicles by epithelial tissue.

4. Similar changes can be produced in fragments of bursa grafted on the chorio-allantoic membrane by prior administration of testosterone to the host embryo.

I. INTRODUCTION

The function of the bursa of Fabricius has become a matter of considerable interest to immunologists in recent years. There is some evidence to suggest that its presence is necessary during the first week after hatching if the chick is to become capable of normal immune responses (Mueller, Wolfe, and Meyer 1960). The bursa begins to atrophy at about 6 months of age and only a fibrous remnant is present at 12 months. There is therefore a tendency to equate its probable function with that of the thymus in mammals especially as the normal histology of the organ is more akin to that of the thymus than any other mammalian organ. From the point of view of the present work a feature of great interest is the process by which the lymphoid follicles of the bursa develop from the early nodules in the pseudo-stratified entodermal epithelium of the early bursal sac. Ackerman and Knouff (1959) believe that there is a direct transformation of proliferating epithelial cells into lymphoblasts from which the lymphocytes of the developed organ eventually derive.

It was first reported by Meyer, Rao, and Aspinall (1959) that administration of about 0.6 mg of 19-nortestosterone at the 4th or 5th day of incubation would completely inhibit the development of the bursa. This has been readily confirmed by Mueller, Wolfe, and Meyer (1960), who showed that "hormonally bursectomized" chickens were sickly after hatching and failed to produce antibody, and by Papermaster, Friedman, and Good (1961), who noted that the hatched chickens were still susceptible (unlike normal birds of the same age) to graft versus host disease when 7-12 days of age.

In view of our general interest in the phenomena of avian immunology we have investigated the possibility of producing birds lacking all lymphoid tissue in the bursa. Since 19-nortestosterone was at the time not available to us, preliminary

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tests were made with testosterone propionate, which was found to have almost identical effects to those described for 19-nortestosterone although the dosage required is higher. In addition to showing the effect obtained by early administration, we have been able to obtain chickens with bursal sacs lined with epithelium but completely lacking lymphoid follicles by giving an appropriate dose of testosterone at 12–13 days incubation. The present paper is concerned with the changes observed in the size and histological appearance of the bursa after administration of testosterone at different stages of development.

II. MATERIALS AND METHODS

Testosterone was obtained as testosterone propionate, 50 mg/ml solution in corn oil (British Drug Houses). Dilutions required were made in sterile olive oil and the dose injected in a volume of 0.1 ml. The yolk-sac route was used in younger embryos, older ones were injected into the allantoic cavity.

Chick embryos were white leghorn/Australorp hybrids from fertile eggs obtained from a commercial source. The eggs before and after injection were incubated at 100°F (37.8°C) and turned daily.

Specimens for histological examination were fixed in Zenker-formol and stained with haematoxylin and eosin.

III. RESULTS

(a) *Normal Development*

The normal development of the bursa of Fabricius in the chick embryo has been described by various authors (see Romanoff 1960), and most recently by Ackerman and Knouff (1960). Our own findings are in full accord with their account.

The general form of the bursa is visible at the 12th or 13th day of incubation when sections show the characteristic plica covered with a uniform layer of pseudo-stratified epithelium. The tunica propria of the organ is composed of loose mesenchymal tissue within which are areas of haemopoietic tissue. About the 13th day points of epithelial proliferation appear and by the following day give rise to nodules of unequivocal epithelium fairly uniformly distributed over the lining membrane. The nodules progressively enlarge, each eventually giving rise to one of the lymphoid follicles (follicles of Stannius) which are present during the weeks following hatching. According to Ackerman and Knouff (1960) the epithelial cells in the nodules proliferate rapidly, lose their normal regular packing, and take on the character of lymphoblasts. From these the lymphoid follicle develops. Earlier observers preferred to believe that the lymphoid transformation was due to the entry of primitive mesenchymal cells from the tunica propria. The general course of development can be clearly seen in Plates 1–3. From the 19th day onward the bulk of the substance of each plica is made up of the lymphoid follicles which soon become closely packed. Each, however, remains clearly demarcated from its neighbours and between the follicles there are strands of connective tissue and blood vessels. This brief account is simply to provide a background for the changes associated with the action of testosterone.

(b) Effect of Testosterone Given during the Early Stages of Development

Following preliminary experiments, two series of 10 or more embryos at each stage of development were injected with 1 or 2 mg of testosterone into the yolk sac. Tests were made from the pre-incubation period onwards but injection of solvent alone produced almost 100% mortality in the earliest stages and results are only significant from 4 days onward. All embryos that had survived were examined at the 19th day of incubation and the macroscopic appearances recorded. Representative bursae were taken for section. The results which are combined in Table 1 indicate that with 2 mg of testosterone there was complete failure of the bursa to appear

TABLE 1

EFFECT OF TESTOSTERONE ON BURSAL DEVELOPMENT OF EMBRYOS TREATED EARLY IN INCUBATION
Findings at 19 days in surviving embryos. 0, no bursa, \pm , bursa very small (one-quarter normal length); +, bursa small (one-half normal length); ++ bursa normal size. Numbers in brackets denote number of embryos with indicated characteristic

Age of Treatment (days)	1 mg Testosterone		2 mg Testosterone	
	No. of Embryos Treated	Findings	No. of Embryos Treated	Findings
4	4	0(2); \pm (2)	7	0(7)
5	5	0(2); \pm (3)	5	0(4); ++(1)
6	9	0(9)	18	0(18)
7	N.T.	—	11	0(6); \pm (5)
8	7	\pm to +(7)	5	\pm (5)
9	7	0(1); \pm (2); +(3); ++(1)	7	\pm (4); +(3)
10	8	+(7); ++(1)	7	+(7)
13	N.T.	—	3	++(3)

in embryos injected at the 6th day or earlier. The one exception at the 5th day must obviously be ascribed to some anomalous situation or technical lapse. Injection on the 7th and 8th days gave a proportion of very small bursae.

It will be obvious from Table 1 that sex of the embryo can have no important bearing on the results. In several series specific note was made of the sex of the embryos being examined at 19 days incubation. There was no indication of any difference in the results as between male and female.

Histologically the very small bursae which developed after early treatment usually showed a small irregular cavity lined by pseudostratified epithelium with at most a few crypt-like structures. No lymphoid tissue was present when 2 mg of testosterone had been used but some of those receiving the smaller dose showed a few small but relatively mature lymphoid follicles (Plate 4, Fig. 1). In the embryos injected at 8 or 9 days various types of abortive development of lymphoid epithelial follicles could be seen. In one embryo treated at 9 days the bursa showed well-developed lymphoid follicles at one side but on the other somewhat polypoid over-

growth of epithelium with very little evidence of nodule or follicle formation (Plate 4, Fig. 2).

Perhaps the most interesting histological findings were those obtained when embryos were inoculated at 13 days, i.e. at a time when development of the initial epithelial stage of the follicles is in progress. When examined at 19 days the bursae were of normal size but of softer consistency than normal, 3-4.5 mm in diameter. Sections showed a much larger lumen lined by irregular folds of epithelium closely resembling normal rectal epithelium. In most sections typical lymphoid follicles were completely lacking. The following notes in regard to epithelial and lymphoid follicles are given for three embryos injected into the yolk sac and six into the allantoic cavity at 13 days:

Embryo No.	Yolk Sac Injection	Embryo No.	Allantoic Cavity Injection
1	One epithelial nodule only	1-5	No epithelial or lymphoid follicles
2	No follicles	6	About 20 epithelial nodules, some showing signs of lymphoid change. Many of these nodules had much of the character of an epithelial crypt and in two instances the nodule showed both a lumen and an area of early lymphoid change
3	12 fairly well-developed lymphoid foci and a few small epithelial nodules showing evidence of change toward lymphoid character		

The nature of such partial replacement of lymphoid follicles by epithelial structures is more clearly seen in bursae from embryos treated at a later stage of development. A test of graded doses of testosterone given at 5 days incubation gave results shown in Table 2.

TABLE 2
TREATMENT AT 5 DAYS INCUBATION WITH GRADED DOSES OF
TESTOSTERONE

0, no bursa; \pm , bursa very small (one-quarter normal length); +, bursa small (one-half normal length); ++, bursa normal size. Numbers in brackets denote number of embryos with indicated characteristic

Dosage per Embryo (mg)	No. of Embryos Treated	Findings at 19 Days	Notes
2	7	0(7)	
1	12	0(9); \pm (3)	Mature lymph follicles in \pm
0.75	6	0(3); \pm (3)	Mature lymph follicles in \pm
0.5	6	+(4); ++(2)	

One milligram per embryo is the smallest dose that can be relied on to produce marked or complete inhibition of development. It was noteworthy that most of the very small bursae that were sectioned showed small numbers of typical lymphoid follicles.

(c) Analysis of Results of Testosterone Treatment at 13 Days

Our primary interest in the effect of testosterone on the bursa of Fabricius was to investigate the effect of eliminating lymphoid tissue in the bursa on various immunological capacities of the chick. The possibility of doing this without producing the side effects of early injection, made it of special interest to investigate the later stages of development in chicks given the hormone at 13 days. At this stage in the normal embryo the bursa has developed its general form but the plicae are covered by a uniform sheet of epithelium within which nodules are just beginning to develop (Plate 1, Fig. 1).

TABLE 3

COURSE OF BURSAL CHANGES AFTER INJECTION OF 2 MG TESTOSTERONE PER EMBRYO AT 13 DAYS INCUBATION

Measurements given are those of the longest diameter of the histological section examined

When Examined	Diameter (mm)	Notes on Histology
After 2 days (15 days incubation)	2.6, 2.2, 2.1	One showed few early follicles, slight activity of epithelium, and increased cellularity
After 5 days (18 days incubation)	2.0, 1.6, 2.9	Largest showed about 20 relatively typical lymphoid follicles. A few small epithelial nodules in another. All show many cells and areas of haemopoiesis
After 8 days (at hatching)	4.4, 3.4, 4.1, 3.2, 3.3	No follicles in two: others small to moderate numbers of poorly formed follicles. Active haemopoietic tissue
After 22 days (14 days old)	2.1, 2.9	Atrophic changes with thickened wall
After 36 days (28 days old)	2.2, 2.6	Similar atrophy in one, moderate polypoid development in the other

Embryos were killed and examined at various stages after injection with testosterone and the main features of the results are shown in Table 3. Since the bursae were as a routine cut longitudinally, the results are comparable.

In summary, this dose does not always completely inhibit the appearance of epithelial nodules and their development toward lymphoid follicles but the numbers are small and in the later stages (after hatching) atrophy of the bursa takes place. This is also shown when the hormone is given at 12 days incubation. Three chickens so treated were killed 3 weeks after hatching. The bursae were small, 2.1, 2.1, and 1.2 mm in longest diameter, with thickened highly cellular walls and a small lumen which in two was composed of incompletely joined clefts. There was no lymphoid tissue visible in any of the sections.

(d) Administration of Testosterone at Later Stages

To complete the picture older embryos were given the same dose, 2 mg per embryo, into the allantoic cavity and sections of the bursa prepared at 19 days incubation and 3 days after hatching. The results are summarized in Table 4.

The most interesting feature of this study is the effect observed on 17-day embryos in which the lymphoid change was well established at the time the testosterone was injected. There has been a striking reversal of the process by which an epithelial rudiment gives rise to a lymphoid follicle. In one way or another the follicles are losing their lymphoid character, the most striking appearance being the appearance of frank epithelial areas which often develop a small vacuole or cyst.

TABLE 4
ADMINISTRATION OF TESTOSTERONE AT LATER STAGES OF DEVELOPMENT

Age of Treatment	Sections Examined at 19 Days Incubation	Sections Examined 3 Days after Hatching
13 days of incubation	Diameters: 4.3, 4.3, 3.8, 3.7, 3.6, 3.1 mm. Rather dilated, some epithelial overgrowth; one with nodules; no typical lymph follicles	Diameters: 3.1, 3.0, 2.5, 2.4 mm. Partial atrophy, with some overgrowth of epithelium in areas. No lymphoid follicles in any section
15 days of incubation	Diameters: 4.0, 3.0, 2.5 mm. These show distorted crypts and epithelial follicles, with only traces of lymphoid follicles in one section	Diameters: 3.9, 2.6, 2.5 mm. Polypoid epithelial growth in two sections. Two show areas with lymphoid epithelial follicles in atypical forms
17 days of incubation	Diameters: 3.7, 3.2, 2.9, 2.9 mm. Well-developed lymphoid follicles but various anomalies of appearance. Epithelial activity with excrescences and proliferation that seems unrelated to original follicles	Diameters: 4.3, 3.0, 3.0, 2.6, 2.5 mm. These show various forms of atrophy and epithelial replacement of lymphoid follicles, with excessive production of epithelial crypts and processes

Often this process seems to go on to the formation of an epithelial crypt in communication with the lumen of the bursa. When, as is usually the case, there is also a polypoid proliferation of epithelium, the end result closely resembles a section of intestinal mucosa. In some instances the picture seems to represent a simple atrophy of the lymph follicles without epithelial change (Plates 6 and 7).

(e) Effect of Testosterone on Bursal Tissue Grafted on the Chorio-allantoic Membrane

As a preliminary to a more intensive investigation of the process by which lymphoid tissue develops in the bursa, a number of experiments were made in which small pieces of bursa from 13-day embryos were implanted on the chorio-allantoic membrane of 14-day embryos. The fragments usually became attached to the membrane and showed some enlargement. They were harvested for section after 5 days further incubation. Results of these experiments in so far as they bear on the effect of testosterone are tabulated (Table 5).

The effects are, in general, what might be expected from the effect of testosterone on the embryo as a whole. On the normal embryo the fragment attaches and undergoes a fairly normal development showing typical lymphoid foci in four out of six and including all which appeared to be "good takes". Two showed considerable epithelial proliferation. The pieces placed on testosterone-treated embryos were considerably larger than when they were placed on the membrane, showed no lymphoid follicles, and an enlarged but relatively simple epithelial coat. There was a much increased amount of loose interstitial tissue. Typical sections are shown in Plate 8, Figures 1 and 2.

TABLE 5

EFFECT OF TESTOSTERONE ON BURSAL TISSUE GRAFTED ON THE CHORIO-ALLANTOIC MEMBRANE

In all cases graft from 13-day bursal fragment

Host	Histology after 5 Days		
	Lymphoid Foci	Epithelial Activity	Enlargement
Normal, 14-day embryo	++ ++ ++ --	++ ++ ± ± ± ±	+ + ± -- --
14-day embryo treated with 2 mg testosterone at day 13	± -- -- -- --	++ ++ ++ ++ + +	++ ++ ++ + +

IV. DISCUSSION

The effect of relatively large doses of testosterone on the development of the bursa of Fabricius is striking and reproducible. At least two actions can be recognized. When the hormone is administered before the 8th day the development of the epithelial sac is inhibited and in a high proportion of embryos no recognizable bursa can be seen macroscopically at the 19th day of incubation. When a bursa does develop it is very small with incomplete development of plicae, but in a considerable proportion of embryos inoculated at 5 days with 1 mg, or less, a small number of relatively normal lymphoid follicles develop. In view of the strongly "lympholytic" action of testosterone at a later stage, one must deduce that the testosterone is metabolized or otherwise rendered inert a few days after injection into the embryo.

The second type of effect is seen characteristically from 13 days incubation onward, i.e. when the process of lymphoid follicle development has been initiated by the appearance of epithelial nodules. All the appearances suggest that the nodules in the epithelium have two possible destinies. They may follow the course normal to the bursa and develop into the lymphoid follicles of Stannius with their very striking resemblance to lobules of thymus with similar demarcation into cortex and medulla. Alternatively they may develop as they would in the large intestine to form epithelial crypts or glandular structures. This is essentially what takes place under the influence of testosterone, and the histological appearances shown particularly in Plates 6 and 7 represent various intermediate stages of the conflict.

DEVELOPMENT OF BURSA OF FABRICIUS

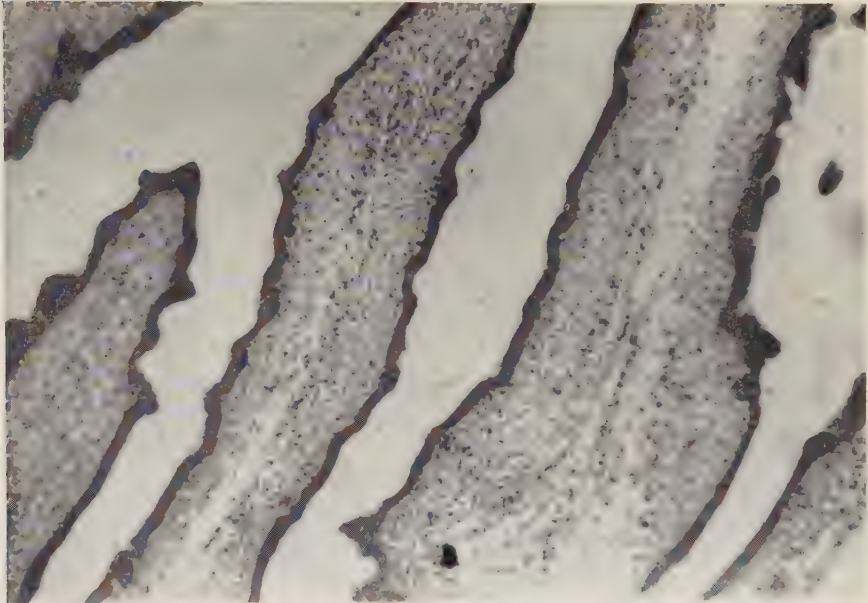


Fig. 1.—Normal bursa of Fabricius at 13th day of incubation. The pseudostratified epithelium covering the plicae shows a few early thickenings. There are areas of haematopoietic tissue in the mesodermal tissue. Haematoxylin and eosin (H and E) stain. $\times 95$.

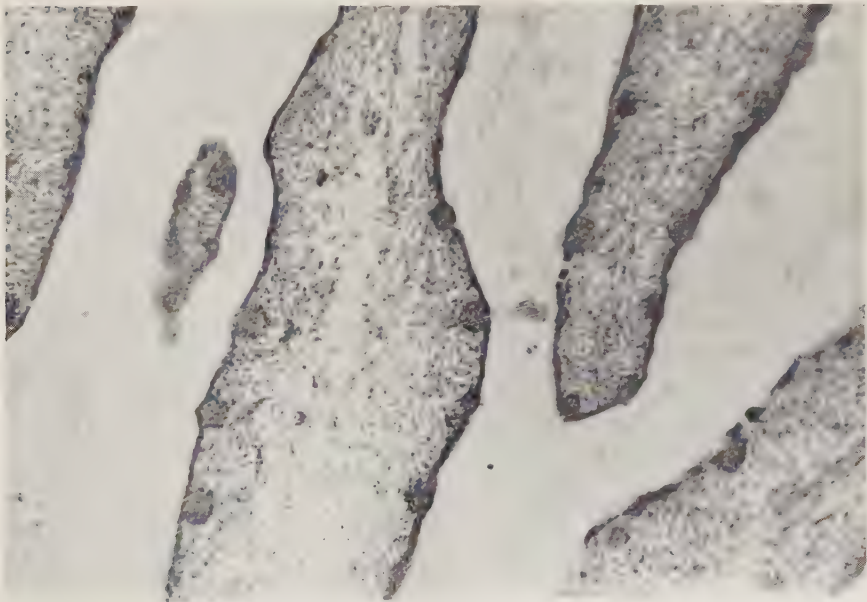


Fig. 2.—Normal bursa at 14 days incubation. The epithelial nodules are clearly developed. H and E stain. $\times 95$.

DEVELOPMENT OF BURSA OF FABRICIUS

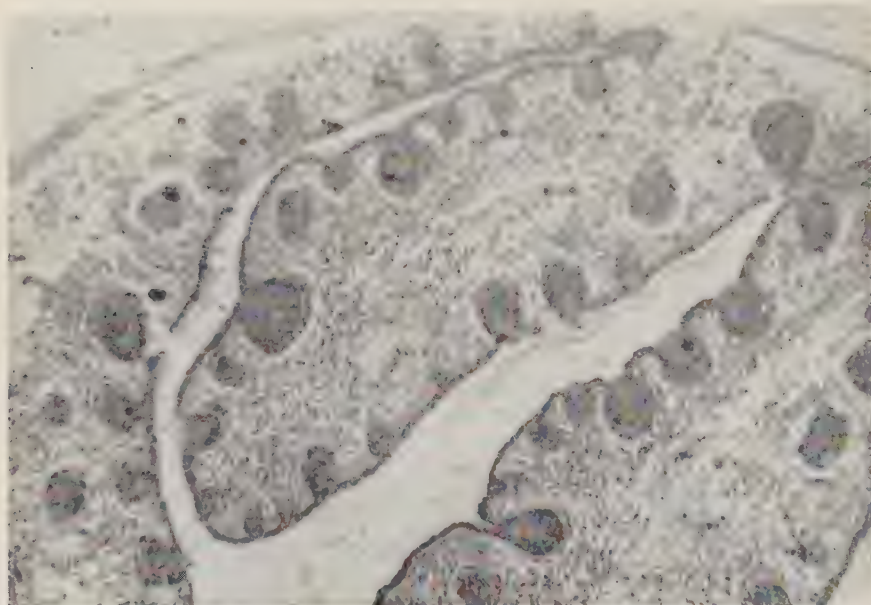


Fig. 1.—Normal bursa after 16 days incubation. Lymphoblastic tissue is developing rapidly in the larger follicles. H and E stain. $\times 95$.

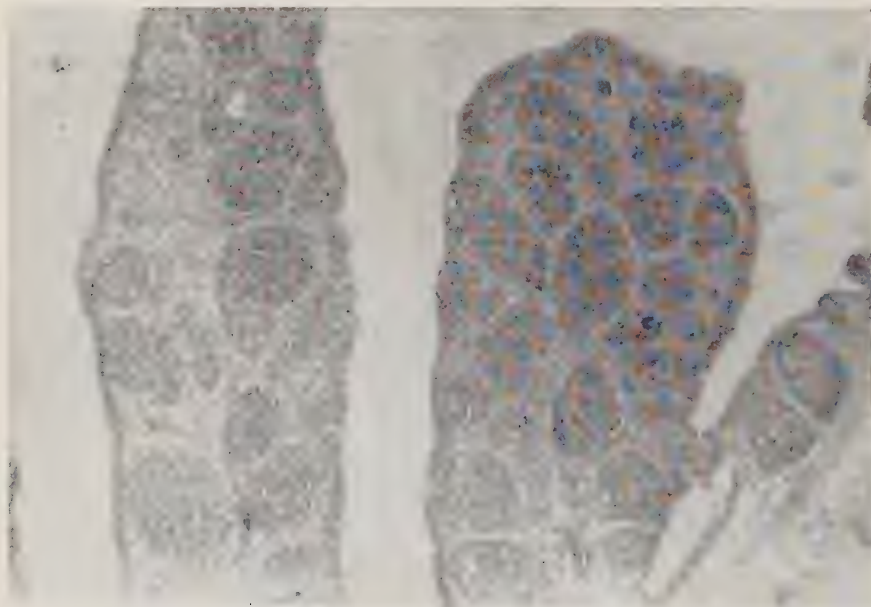


Fig. 2.—Normal bursa after 19 days incubation. The lymphoid follicles are large and have begun to crowd out the interstitial tissue. H and E stain. $\times 95$.

DEVELOPMENT OF BURSA OF FABRICIUS

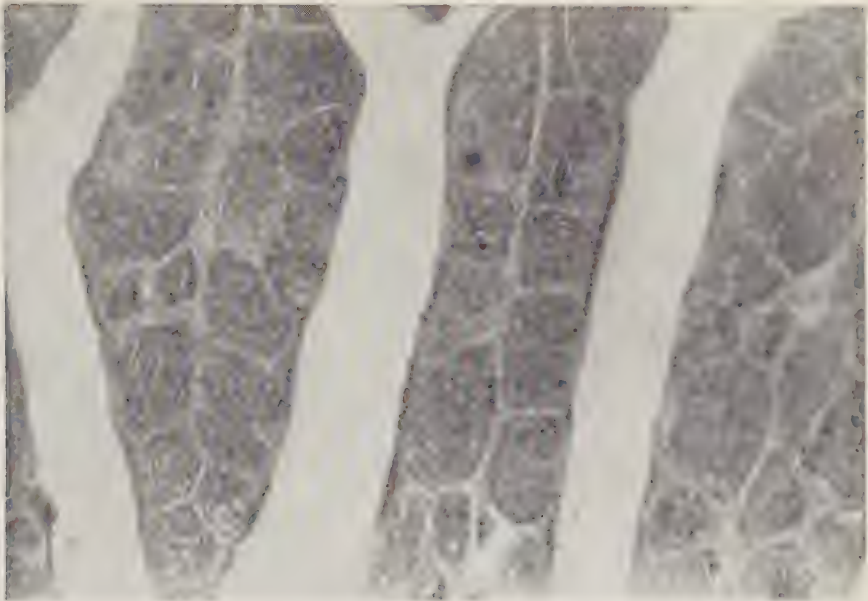


Fig. 1.—Normal bursa 2 days after hatching. Follicles now almost fill the plicae. H and E stain. $\times 95$.

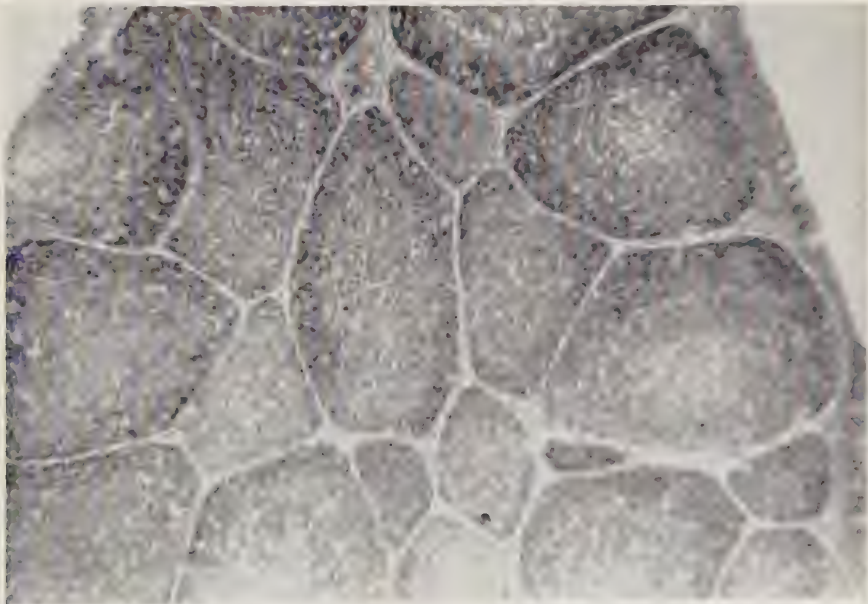


Fig. 2.—Fully developed bursa of Fabricius from a normal 3-months-old chicken. The follicles are sharply demarcated in a thymus-like distribution having a darkly staining cortex and a lighter central medulla. H and E stain. $\times 95$.

DEVELOPMENT OF BURSA OF FABRICIUS

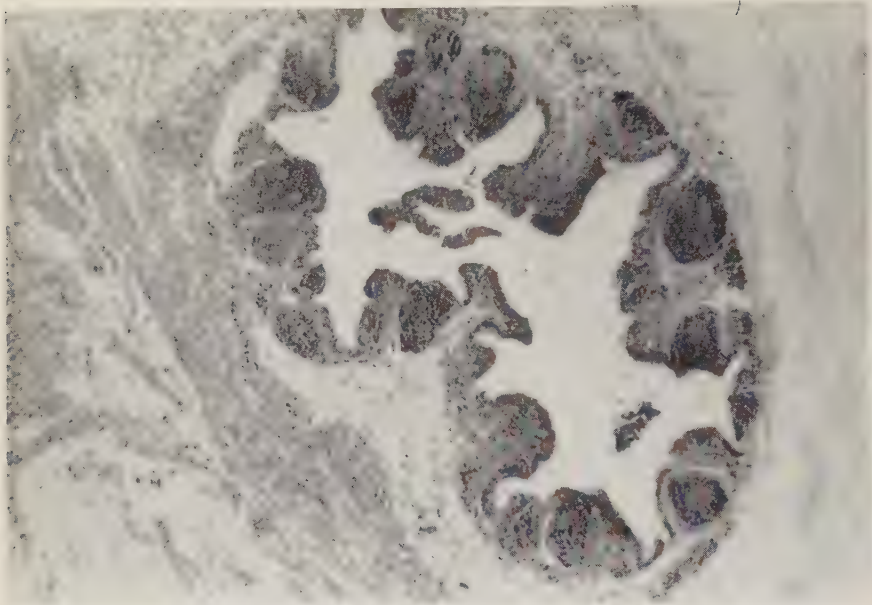


Fig. 1.—Testosterone (1 mg) given at 5 days; section at 19 days showing atrophic bursa with a few relatively normal lymphoid follicles. H and E stain. $\times 95$.

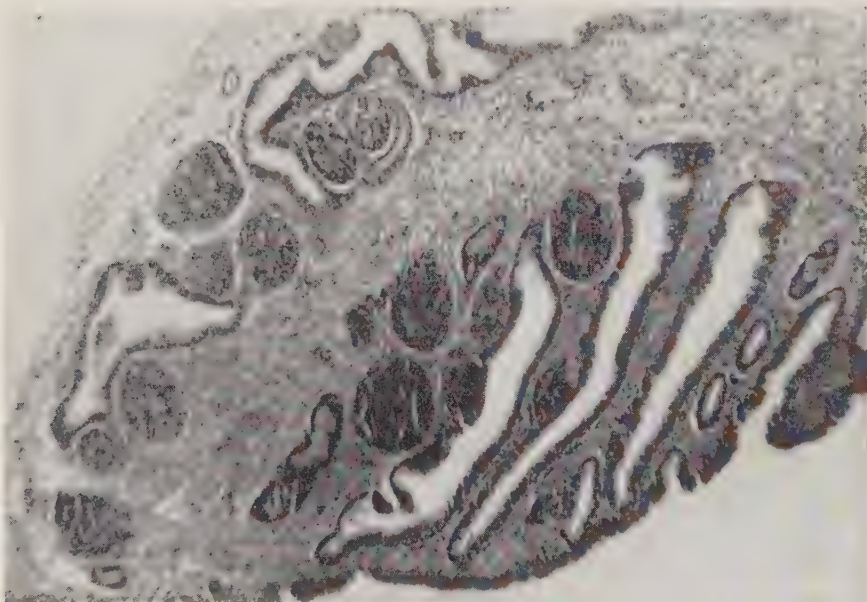


Fig. 2.—Testosterone (2 mg) given at 9 days; section at 19 days showing normal lymphoid follicles with various types of epithelial overgrowth. H and E stain. $\times 95$.

DEVELOPMENT OF BURSA OF FABRICIUS

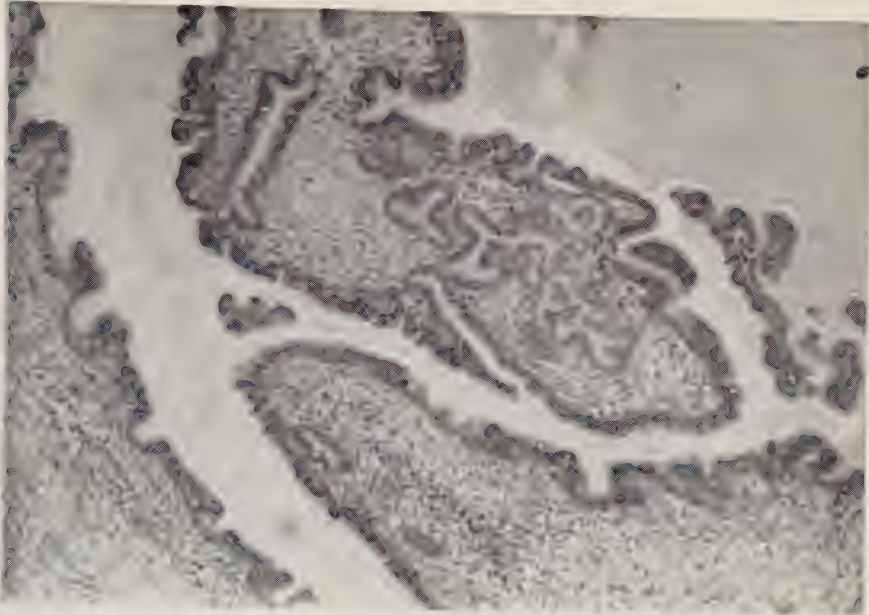


Fig. 1.—Testosterone (2 mg) at 13 days; section at 19 days. Showing epithelial activity with crypts and polypoid overgrowth. No sign of lymphoid follicles. H and E stain. $\times 95$.

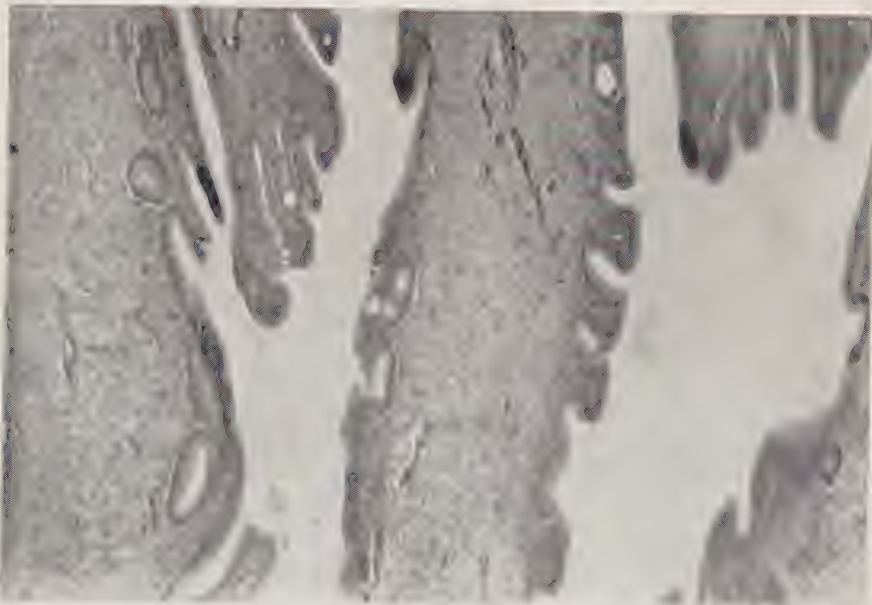


Fig. 2.—Testosterone (2 mg) at 12 days; section 3 weeks after hatching. Showing fibrous change in interstitial tissue with moderate epithelial proliferation: no lymphoid tissue. H and E stain. $\times 95$.

DEVELOPMENT OF BURSA OF FABRICIUS

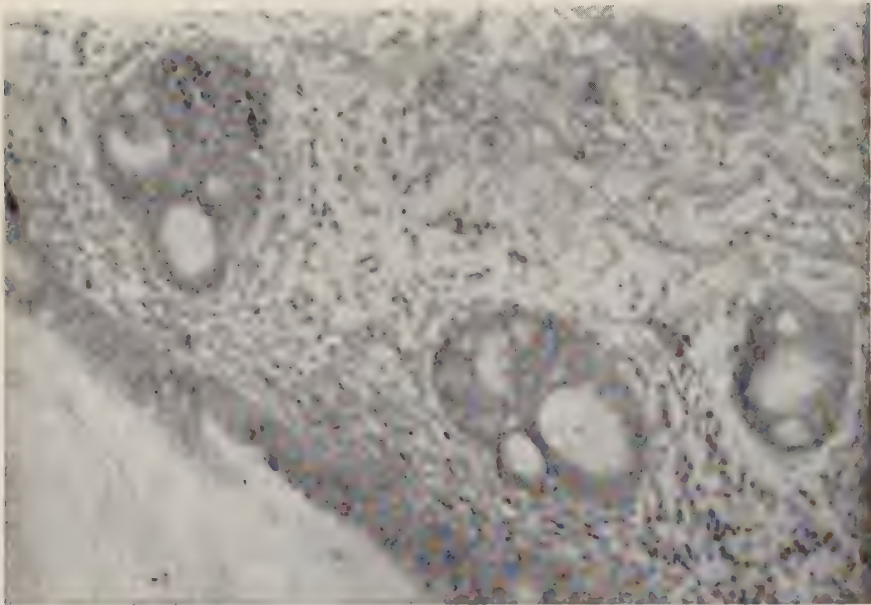


Fig. 1.—Testosterone (2 mg) given at 15 days; section at 19 days incubation. This section shows three converted lymphoid follicles, the largest still showing an area of lymphoid tissue. In all three, epithelial cells surround a system of cystic cavities. H and E stain. $\times 240$.

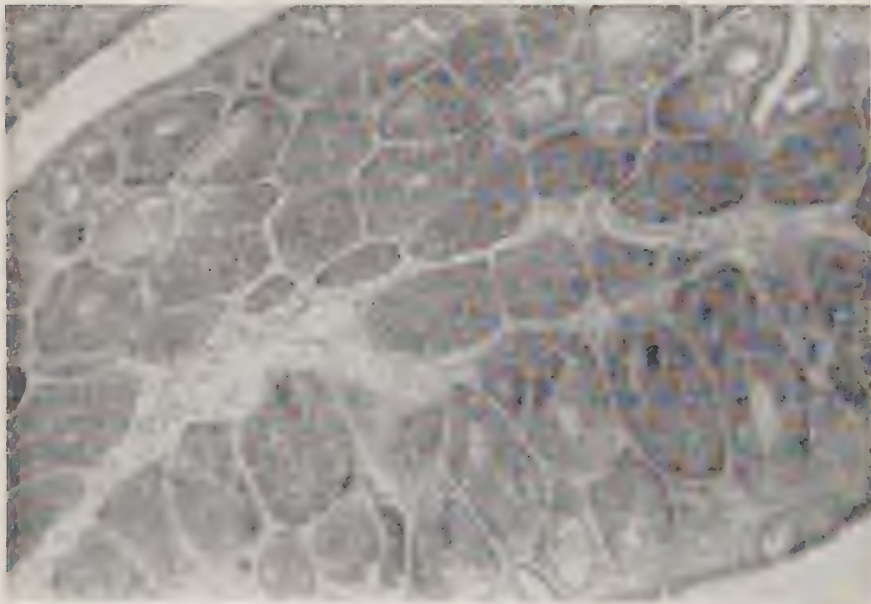


Fig. 2.—Testosterone (2 mg) given at 17 days; section 3 days after hatching. Showing relatively slight epithelialization in almost fully developed lymphoid follicles. H and E stain. $\times 95$.

DEVELOPMENT OF BURSA OF FABRICIUS

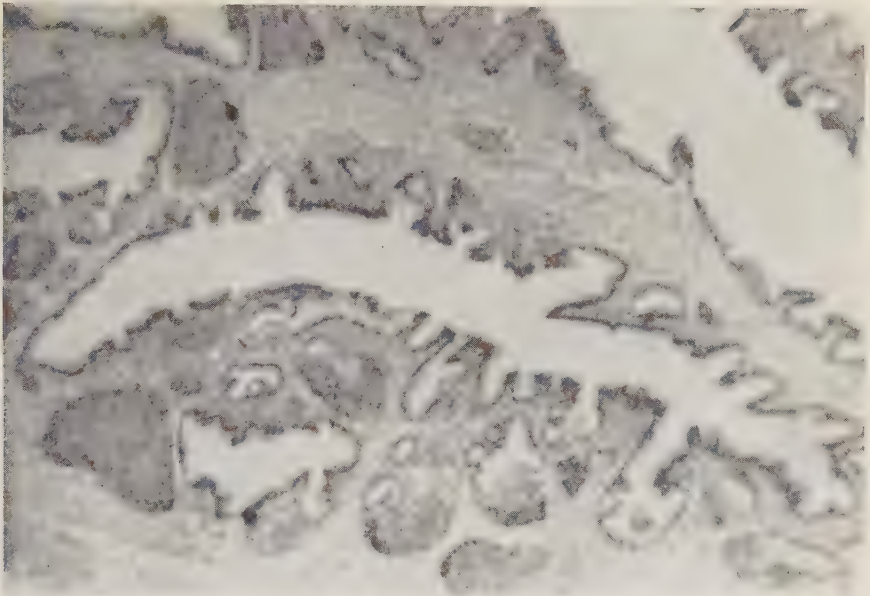


Fig. 1.—Testosterone (2 mg) given at 17 days; section 3 days after hatching. The section shows some large intact lymphoid follicles. Most, however, have been replaced either completely by epithelial cysts or crypts, or show various degrees of such conversion to epithelial structures. H and E stain. $\times 95$.

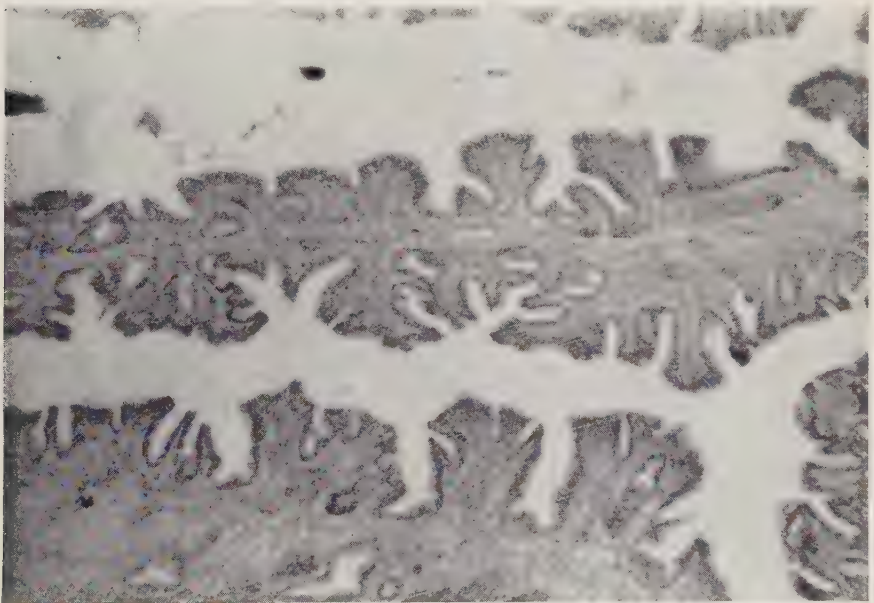


Fig. 2.—Testosterone (2 mg) given at 16 days; section 3 days after hatching. Showing complete replacement of follicles by crypts with polypoid growth of epithelium. H and E stain. $\times 95$.

DEVELOPMENT OF BURSA OF FABRICIUS

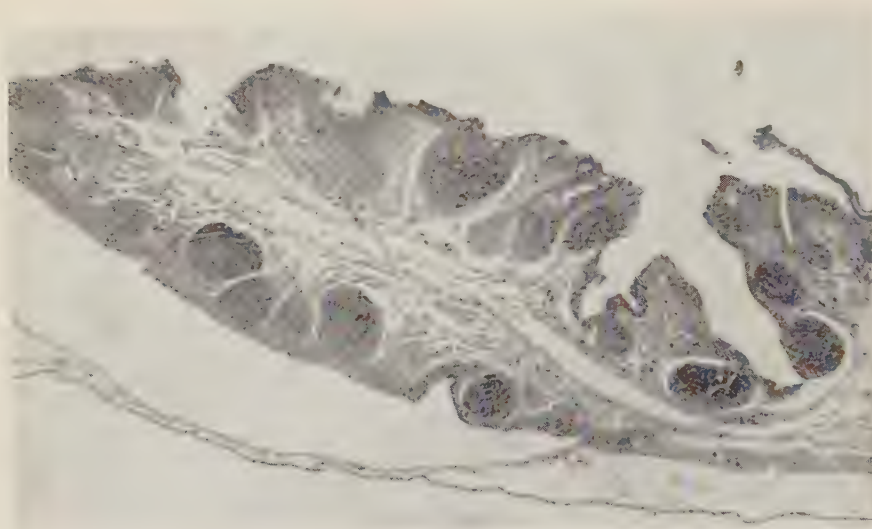


Fig. 1.—Graft of 13-day bursal tissue on the chorio-allantoic membrane of normal 14-day embryo. Section made after 5 days on the chorio-allantoic membrane and shows essentially normal development of lymphoid follicles. H and E stain. $\times 95$.

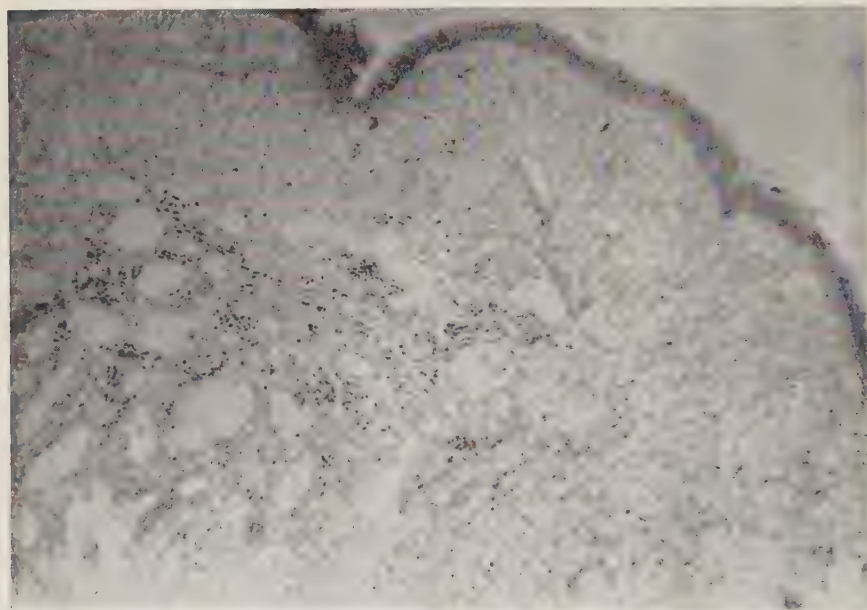


Fig. 2.—Graft of 13-day bursa on 14-day chorio-allantoic membrane of embryo given 2 mg testosterone on the 13th day of incubation. Section made after 5 days on the chorio-allantoic membrane and shows active growth of mesodermal tissues and surface epithelium with a few crypts but no trace of lymphoid follicles. H and E stain. $\times 95$.

A closer analysis of the process will need much more attention to histochemical detail than has yet been possible. Superficially our results speak strongly in favour of the epithelial origin of the follicles. The changes that follow testosterone treatment of 17-day embryos seem to represent a direct transformation of medullary cells into epithelium of intestinal type. However, it would be equally possible for those who believe that the lymphoid cells are derived from mesenchymal cells invading the epithelial nodules from the interstitial tissue to provide a corresponding explanation for the reverse change. On this view the medulla contains, in addition to lymphoblasts and reticulum cells, discrete cells of epithelial origin. Under the influence of testosterone these are stimulated to proliferate and as they do, to crowd out the lymphoid cells which may either autolyse or pass into the blood stream. A decision between these two alternatives may have an important bearing on the process of differentiation by which lymphocytes are produced.

We are not concerned in this paper with the immunological significance of the bursa or the effect of its functional absence on the health of the hatched chicken. These features are under study and it need only be noted that chickens "hormonally bursectomized" by early administration suffer from impacted faeces and usually die early. This confirms earlier findings of Mueller, Wolfe, and Meyer (1960) and Papermaster, Friedman, and Good (1961). Those treated later (12-13 days) show better survival though there is still an abnormally high mortality.

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INHERITANCE OF ANTIBODY RESPONSE

IV. HERITABILITY OF RESPONSE TO THE ANTIGENS OF RHIZOBIUM MELILOTI AND TWO STRAINS OF INFLUENZA VIRUS

By W. R. SOBEY* and K. M. ADAMS*

[Manuscript received June 16, 1961]

Summary

A heritability of -0.012 is obtained in mice to injections of *Rhizobium meliloti* as measured by the Vi antigen and 0.78 by the O antigen.

The heritability of antibody response to injections of influenza virus strain MEL is 0.60 and 0.16 and to strain LEE 0.30 and 0.10 when measured by two methods.

These differences are discussed and suggestions offered to account for low heritabilities.

I. INTRODUCTION

The inheritance of the amount of antibody produced in response to injections of antigens has been studied by Sang and Sobey (1954), Sobey (1954), Sobey and Adams (1955), and Claringbold, Sobey, and Adams (1957).

Sobey and Adams (1955) suggested that low heritabilities of antibody production in response to injections of complex antigens could be expected if the titre recorded was that of the component antibody in highest concentration. Measurement of the titre of individual antibodies or measurements of the total of all antibodies present was necessary for accurate determination of heritability of antibody production.

Two antigens were recommended for further investigation, *Rhizobium meliloti*, containing two antigens, and influenza virus. Influenza virus was suggested as it was believed that the technique of measuring neutralizing antibody to influenza virus would measure one specific antibody only (Fazekas de St. Groth, personal communication).

The use of these antigens together with an improved design of experiment for measurement of heritability (Claringbold, Sobey, and Adams 1957) was expected to give additional useful information about this problem.

II. MATERIALS AND METHODS

(a) Antigens

Rhizobium meliloti, strain "Sydney University 277/1" and the mouse-adapted strains of the two influenza virus strains MEL and LEE were used in this investigation.

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(b) *Serological Techniques*

(i) *Rhizobium meliloti*.—This organism contains a Vi-like and an O antigen. It has been demonstrated (Vincent 1953) that the Vi antigen of *Rhizobium*, unlike that of *Salmonella typhosa*, does not completely inhibit the demonstrable O antigen-antibody reaction when unheated antigen is used in testing, though some inhibition may be experienced. For this reason, the antigen was used unheated for measuring response to the Vi antigen and heated at 100°C for 30 min to destroy the Vi antigen when measuring response to the O antigen.

A stock suspension of the organism was kept at a concentration of 5×10^8 organisms/ml.

Methods of bleeding and separating of serum were as described by Sobey and Adams (1955). Serum was not inactivated before testing. Using a pipette drawn to deliver 30 drops/ml, two drops of serum were delivered serially into precipitin tubes containing two drops of calcium-magnesium saline, thus diluting the serum twofold at each step. One drop of antigen was added to each tube, the tubes then incubated in a water-bath at 37°C for 4 hr, left at room temperature overnight, and the reaction recorded the following morning. Readings were made in a specially constructed and illuminated black box with the aid of a hand lens.

The end-point was found to be clear cut. The relative agglutination titre of a serum was recorded on a log scale simply by taking tube number as the titre values. Titres of antibodies to the Vi and O antigens were separately measured on each antiserum. In order to choose the best dose of antigen for injection and the best bleeding time, the following factorial experiment was undertaken, measuring the titre of antibodies to the O antigen only. The antigen was given at three levels, 1 ml of 5×10^8 , 5×10^7 , and 5×10^6 organisms/ml, in two intravenous injections of 0.5 ml; each spaced by 4 days. The mice were bled 3½, 7, 10½, 14, and 17½ days after the last injection. All the mice used came from a randomly bred albino stock maintained in this Laboratory. Mice were housed four to a box and supplied mouse cubes and water *ad lib*. Four mice were allocated at random to each treatment group. This gave a 3×5 factorial with four mice per treatment group, making a total of 60 mice. The design, results, and the analysis of variance are given in Table 1.

Antibody titre was seen to increase log-linearly with dose. Higher titres were recorded at the bleeding time of 7 days and with the dose of 5×10^8 organisms/ml.

(ii) *Influenza Virus Strain MEL and LEE*.—Methods of bleeding and separating of serum were as described in Sobey and Adams (1955).

Antisera produced in response to injections of both strains of virus were measured for antihaemagglutinin and neutralizing antibody.

Measurement of antihaemagglutinin was done in plastic trays. After inactivation and destruction of non-specific inhibitions by R.D.E-citrate-65°C treatment (Fazekas de St. Groth 1949), twofold dilutions were made up with Takatsy loops in 0.25 ml saline. A standard drop (0.025 ml) of 5% fowl cells was then added, and finally a drop containing exactly 4 agglutinating doses of the test virus. The trays were shaken, and the pattern of cells read after half an hour at room temperature.

Relative titres were expressed as the log of the antihaemagglutinin titre by taking well numbers from the plastic trays as titre values.

Neutralization tests were carried out in plastic trays using the method described by Fazekas de St. Groth, Withell, and Lafferty (1958). Titres were expressed as the "mean neutralizing potency" or pN value, a score derived by Fazekas de St. Groth (1961).

TABLE 1
DESIGN, RESULTS, AND ANALYSIS OF VARIANCE OF THE RHIZOBIUM DOSE-TIME
RESPONSE EXPERIMENT

Bleeding Times (days after last injection)	Dose of <i>Rh. meliloti</i> (2×0.5 ml injections, 4 days between injections)			
	5×10^8 /ml	5×10^7 /ml	5×10^6 /ml	Totals
$3\frac{1}{2}$	7	4	0	
	8	7	0	
	8	2	0	
	1	2	0	
Totals	24	15	0	39
7	10	8	5	
	9	9	4	
	10	7	0	
	9	9	0	
Totals	38	33	9	80
$10\frac{1}{2}$	5	0	5	
	6	0	5	
	8	0	0	
	0	8	3	
Totals	19	8	13	40
14	7	7	0	
	8	2	5	
	2	2	3	
	2	0	0	
Totals	19	11	8	38
$17\frac{1}{2}$	4	4	0	
	10	7	0	
	9	4	0	
	6	0	2	
Totals	29	15	2	46
Grand totals	129	82	32	243

TABLE 1 (*Continued*)
Analysis of Variance

Source of Variation	Degrees of Freedom	Mean Square	F
Doses	(2)		
Linear	1	235.2	35***
Deviations	1	0.1	1
Bleedings	(4)		
Linear	1	6.5	1
Quadratic	1	4.7	1
Cubic	1	69.0	10**
Quartic	1	25.7	3.8
Interactions	8	10.1	1.4
Error	45	6.7	

** $P < 0.1$. *** $P < 0.01$.*(c) Design of Heritability Experiments*

(i) *Rhizobium meliloti*.—128 daughter/dam pairs of adult albino mice weighing about 25 g, obtained from a randomly bred stock maintained in this Laboratory, were used. The dams were housed in 32 boxes, and they and their daughters were prepared as for an earlier experiment (Claringbold, Sobey, and Adams 1957). For convenience, the experiment was divided into four blocks (*BL*). Each block comprised 32 boxes of dams (*D*) and 32 corresponding boxes of daughters (*d*). The mice were given standard mouse cubes and water *ad lib*.

1 ml 5×10^8 organisms/ml was given in two 0.5 ml intravenous injections spaced by 4 days. Mice were bled 7 days after the last injection. The treatment of successive blocks was commenced at weekly intervals but was otherwise identical.

(ii) *Influenza Virus*.—The same animals and design were used for this determination. Mice were injected with each of the two virus preparations simultaneously, 0.25 ml of both MEL and LEE were administered by the intraperitoneal route. Mice were bled 14 days after injection for MEL antibody titration and 18 days after injection for LEE titration. The first two blocks of animals were injected together and the second two blocks were injected 21 days later.

III. RESULTS

Heritabilities were obtained by doubling the correlation of daughter with dam scores. The results of the *Rhizobium* titrations are presented in Table 2. A heritability of -0.012 for Vi and 0.78 for O antigen was obtained.

The results of the influenza virus titrations by both antihaemagglutinin and neutralizing potency tests are presented in Table 3. Heritabilities of 0.60 (0.43 – 0.74) by antihaemagglutinin and 0.16 (0.1 – 0) by the neutralizing potency test were obtained for MEL virus, and heritabilities of 0.30 (0.06 – 0.51) by antihaemagglutinin and 0.10 (0.1 – 0) by the neutralizing potency test were obtained for LEE virus.

IV. DISCUSSION

Methods of measuring antibody response are probably open to limitations where more than one antibody is concerned. By serial dilution of antiserum, antibodies with low titres will become too low in concentration to contribute to the reaction, and the end-point giving the measure of response will be determined by the

TABLE 2
VARIANCE AND COVARIANCE FOR THE RHIZOBIUM TITRATIONS

Antigen	Variance Dam	Variance Daughter	Covariance	r	h^2	95% Limits
Vi	222.43	363.56	-17.76	-0.006	-0.012	—
O	407.74	329.47	138.88	0.39	0.78	0.13-0.90

titre of the antibody which is in greatest concentration. It is unlikely that the different antibody titres are additive in respect of the end-point, the consequence being that in no instance would total antibody response be measured; the measure would be of antibody in highest concentration. This has been shown to be true of the A, B, and O human antigens (Sobey and Adams 1955). Since the same antibody would not necessarily be present in greatest concentration in all individuals, varia-

TABLE 3
VARIANCE AND COVARIANCE FOR THE INFLUENZA VIRUS TITRATIONS

Virus Strain	Test*	Variance Dam	Variance Daughter	Co- variance	r	h^2	95% Limits
MEL	N	1531	297	56	0.08	0.16	0-1
MEL	A	7603	7935	2335	0.30	0.60	0.43-0.74
LEE	N	1280	1555	75	0.05	0.10	0-1
LEE	A	7130	4399	823	0.15	0.30	0.06-0.51

* N = neutralizing potency test; A = antihaemagglutinin test.

tion observed would not be variation of a single specific antibody and this would result in an apparent lack of genetic variability. For example, when testing for response to Vi of *Rhizobium*, both the Vi and O antigens are present, and heritability is low, as demonstrated from Table 2; this is not surprising and is in accord with previous arguments. When the Vi antigen is destroyed by heating, the only measurable response is that to the O antigen and here the heritability of response is high, $h^2 = 0.78$, and is close to that found for tobacco mosaic virus by Sang and Sobey (1954), where apparently a single antibody response is measured.

From the influenza virus data in Table 3, low heritabilities are recorded for both strains of virus as measured by the neutralizing potency test, whilst both strains give a higher heritability as determined by antihaemagglutinin titration. If the neutralizing potency test did measure only a single response, as then believed, then, on the assumption of a high degree of genetic control of individual antibody responses, no valid explanation could be put forward to support the low heritability obtained. Further work by Lafferty on this problem showed that the neutralization test measured not a single antibody but a complex of antibodies (Lafferty, unpublished data).

It has been shown by Claringbold (unpublished data, 1961), using the above assumptions in Monte Carlo runs in the electronic digital computer SILLIAC, that with increasing numbers of antigenic components, each with a heritability of 100% and no correlation, the measured heritability decreases such that with 10 antigens the heritability is 50%. Further, where one antigen is included in the model with a low heritability the measured heritability approaches this value regardless of the heritability values attributed to the other antigenic components in the model. These results from the computer do not include any environmental variation, which in actual observations would further lower heritability.

It is suggested that these findings support previous arguments concerning the complexities of antigens and responses to them, and that still more studies on the inheritance of antibody response are required using purified antigens which will elicit a single antibody response or using some means to identify and measure an individual response from within a complex response pattern.

V. ACKNOWLEDGMENTS

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INHERITANCE OF ANTIBODY RESPONSE

V. CORRELATED ANTIBODY RESPONSES TO VARIOUS RELATED AND UNRELATED ANTIGENS

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[*Manuscript received June 16, 1961*]

Summary

A number of correlation coefficients of responses of antibody injections of like and unlike antigens are presented. There is a suggestion that there is present a predisposition to immunity. The findings of negative correlations are discussed in view of suggestions of error of measurement of responses.

I. INTRODUCTION

For some time past it has been known that animals of the same species show a marked variation in their responses to a given antigen.

Experiments have been carried out in the past (Carlinfanti and Cavalli 1945, 1947; Carlinfanti 1947, 1950; Sang and Sobey 1954) on the problem of predisposition for immunity. Carlinfanti (1947) states that a correlation does exist among the titres of antibodies produced by one subject in response to different antigens. He suggests that this correlation decreases, however, until it reaches a value not significant from zero, when antibodies of different types are considered.

The importance of such a predisposition, if real, is great and has great bearing on the whole problem of immunity.

Sang and Sobey (1954) examined the genetic features which control the response of antibodies and have stressed the importance of genetic constitution in determining ability to produce antibodies.

The experiments to be described, using related and unrelated antigens in mice and rabbits, gave information to help in the clarification of this problem.

II. MATERIALS AND METHODS

(a) *Antigens for Mice*

Rhizobium meliloti, strain "Sydney University 277/1" and the mouse-adapted strains of influenza virus MEL and LEE were used in this investigation.

Mice were given 5×10^8 rhizobial organisms/ml, in two 0.5 ml intravenous injections spaced by 4 days. They were bled 7 days after the last injection.

The two virus preparations were injected simultaneously; 0.25 ml each of strains MEL and LEE were administered by the intraperitoneal route. Mice were bled 14 days after the last injection for the MEL titration and 18 days after injection for the LEE titration.

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(b) *Antigens for Rabbits*

Influenza virus strains MEL and LEE, vaccinia virus, tobacco mosaic virus (TMV), and potato X virus were used.

Rabbits were injected intravenously with 0.25 ml of each of the influenza virus preparations simultaneously. They were bled as described for mice injected with this preparation.

Two intravenous injections of 1 ml, each containing 0.5 mg TMV and 0.25 mg potato X virus, were given, spaced by 1 week, to rabbits which were bled 10 days after the last injection.

Vaccinia virus was administered in one 0.5 ml intravenous injection and rabbits were bled 10 days later.

TABLE 1

CORRELATION COEFFICIENTS OF RESPONSES TO VARIOUS ANTIGENS IN MICE

MEL, LEE represent influenza strains MEL and LEE respectively, and O, Vi represent the O and Vi antigens of *Rh. meliloti*. N = neutralizing potency titre; A = antihaemagglutinin titre

No.	Antigen Responses Correlated	Correlation Coefficient*	Degrees of Freedom	No.	Antigen Responses Correlated	Correlation Coefficient*	Degrees of Freedom
1	MEL(N)–LEE(N)	0.51	201	8	O–MEL(N)	–0.09	216
2	MEL(A)–LEE(A)	0.32	194	9	O–MEL(A)	–0.10	212
3	MEL(N)–MEL(A)	0.59	212	10	O–LEE(N)	–0.04	212
4	LEE(N)–LEE(A)	0.71	212	11	O–LEE(A)	0.10	208
5	MEL(N)–LEE(A)	0.47	198	12	Vi–MEL(N)	–0.02	216
6	MEL(A)–LEE(N)	0.29	198	13	Vi–MEL(A)	–0.04	212
7	O–Vi	0.34	256	14	Vi–LEE(N)	–0.01	212
				15	Vi–LEE(A)	–0.03	208

* S.E. = 0.14.

(c) *Serological Techniques*

(i) *Rhizobium meliloti*.—The method of detection of antibodies to this organism, containing a Vi-like and O antigen, are described in detail in Part IV of this series (Sobey and Adams 1961).

(ii) *Influenza Virus*.—Responses in mice to injection of both strains of this virus were measured for antihaemagglutinin and neutralizing titre, whilst in rabbits antihaemagglutinin titre only was measured. Methods of measurement were as previously described (Sobey and Adams 1961).

(iii) *Vaccinia Virus*.—Production of antibodies to this virus was measured by the antihaemagglutinin method.

(iv) *TMV and Potato X Virus*.—Titration of antisera were measured by the equivalence zone method as described by Sang and Sobey (1954).

(d) *Experimental Animals*

(i) *Mice*.—128 daughter/dam pairs of adult albino mice weighing about 25 g, obtained from a randomly bred stock maintained in this Laboratory, were used. The mice were prepared as for an earlier experiment (Claringbold, Sobey, and Adams 1957). For convenience, the experiment was divided into four blocks. Each block comprised 32 boxes of dams and 32 corresponding boxes of daughters. The mice were given standard mouse cubes and water *ad lib*.

(ii) *Rabbits*.—50 randomly bred adult rabbits of both sexes weighing about 2.5 kg were used. The rabbits were given standard rabbit pellets and water *ad lib*.

TABLE 2

CORRELATION COEFFICIENTS OF RESPONSES TO VARIOUS ANTIGENS IN RABBITS

TMV = tobacco mosaic virus; X = potato X virus; V = vaccinia virus; MEL, LEE = influenza virus strains MEL and LEE respectively

No.	Antigen Responses Correlated	Correlation Coefficient*	Degrees of Freedom	No.	Antigen Responses Correlated	Correlation Coefficient*	Degrees of Freedom
1	TMV-X	0.35	50	6	MEL-LEE	0.97	50
2	TMV-MEL	0.53	50	7	MEL-V	0.14	50
3	TMV-LEE	0.62	50	8	LEE-V	0.14	50
4	X-MEL	-0.17	50	9	TMV-V	0.21	50
5	X-LEE	-0.09	50	10	X-V	-0.21	50

* S.E. = 0.16.

III. RESULTS

The correlation coefficients of the responses to various antigens tested in mice are shown in Table 1. It is seen that there are strong correlations between responses to like antigens whilst weak or negative correlations are found between responses to unlike antigens; however, the like antigens are two strains of the same virus.

Correlation coefficients of the responses to antigens tested in rabbits are shown in Table 2. No regular pattern of correlation seems to appear from these data.

For example, the animal influenza and vaccinia viruses have weak correlated responses, one with the other, yet between TMV, a plant virus, and the influenza viruses there is a strong correlation. Correlation between potato X and vaccinia or potato X and influenza viruses is of a negative value.

It is of importance to know if an animal which is a good responder to a particular antigen is likely to be a good responder when challenged with another type of antigen, even if the antigens be unrelated.

The total variance found in these two experiments can be apportioned into that found within and between animals. If the latter exceeds the former, as

measured by the usual F test, it can be taken that responses to the antigens are unlikely to be randomly distributed and hence that individual animals tend to produce a similar type of response to each antigen. This calculation is derived from the formula given by Sang and Sobey (1954). The difference between mice gives $F_{210-2940} = 3.75$ and between rabbits gives $F_{49\ 441} = 3.3$. These significant results suggest a predisposition to immunity in both mice and rabbits.

IV. DISCUSSION

The overall results from both mice and rabbits suggest from positive correlations that there may be a pattern in correlation of response between like antigens whilst unlike antigens give weak or negative correlations.

Suggestion of a predisposition to immunity and the likelihood of individuals producing similar responses to antigens would make it of interest to continue and expand this study to include antigens of similar molecular weight, protein pattern, or origin.

If the arguments advanced by Sobey and Adams (1955, 1961) that there is an error of measurement of response due to antigen complexity resulting in formation of a diverse family of antibodies, is correct, then the absence of an observed correlation does not necessarily rule out a predisposition to immunity. A positive correlation, however, is presumably real since information can be taken from but not added to data.

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THE DISTRIBUTION OF LARVAE OF RANDOMLY MOVING INSECTS

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Summary

Though randomly moving insects released from a central point in a uniform environment are often found to be distributed according to a circular normal distribution, their larvae will not conform to this distribution. When such insects lay at a constant rate and are subject to constant mortality, their larvae are found to be spatially distributed according to a highly peaked frequency function, depending on the modified Bessel function of the second kind. This theoretical conclusion is in good agreement with published data. Some of the properties of the theoretical distribution are discussed.

I. INTRODUCTION

In studying the movement of wild populations we commonly assume that each individual moves randomly and independently of the other individuals, with the same distribution of velocities (the random walk). Under such simple assumptions, the distribution of individuals at any time after release from a central point may be readily determined. As might be expected from the similarity of assumptions made about the two phenomena, the equations governing the random movement of organisms are the same as those for diffusion in a homogeneous medium. Random walk is usually studied in one or two dimensions, but diffusion may be studied in one, two, or three dimensions.

In the two-dimensional random walk, with the population initially concentrated at a point source, which we take as the origin, the distribution at time t may be expressed as

$$(1/\sigma^2 t) \exp(-r^2/2\sigma^2 t) r dr.$$

Here r is the distance from the point of release, and σ^2 is the variance of the spatial distribution per unit of time. Thus the variance is seen to be proportional to t , and the linear extent of the distribution to be proportional to \sqrt{t} .

This is the circular normal distribution, which arises also from the diffusion equation

$$\frac{\partial F}{\partial t} = \frac{1}{2}\sigma^2 \left(\frac{\partial^2 F}{\partial x^2} + \frac{\partial^2 F}{\partial y^2} \right).$$

Many phenomena arising from the random movement of animals do not, however, follow the normal distribution. We consider here the distribution of the infestation of fruit by the larvae of the codling moth. Since the female lays eggs continuously throughout its life, the distribution of larvae will consist of a mixture of the circular normal distributions representing the spatial distributions of the moths

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at different times. Only under very special conditions will such a "compound distribution" be itself normal. We here consider a compound distribution which appears to represent satisfactorily the distribution of infestation of fruit by larvae.

Interest in this question has recently been aroused by some experimental work published by Wildbolz and Baggiolini (1958). By releasing a large number of moths virtually at a point in a fairly uniform orchard, they were able to study the effects of the random movement and egg laying of the moths through assessing the numbers of fruit infested with the larvae emerging from the eggs.

Their interesting interpretation of the data has been supplemented by the work of Geier (unpublished data, 1961), who shows that the observed distribution of infested fruit is well enough fitted by a Bessel function with suitably chosen constants.

II. DERIVATION OF THE BASIC DISTRIBUTIONS

It is the purpose of this paper to give a mathematical derivation of the Bessel function distribution, and to suggest conditions under which it may be expected to hold.

Assumption 1:

- (a) Moths are equally mobile, and move independently at random.
- (b) Each female lays eggs at a constant daily rate, λ .
- (c) The moths have equal chance of death in any period, the mortality rate per day being μ .

Other sets of assumptions will be considered later.

For a population subject to these conditions, the average number of eggs laid per female is

$$\lambda \int_0^{\infty} \exp(-\mu t) dt = \lambda/\mu.$$

Now owing to the random movement of the moths, the proportion of moths at a distance r or more from the origin at time t is $\exp(-r^2/2\sigma^2 t)$. Hence, in the interval $t, t+dt$, for each moth the expected number of eggs laid at a distance greater than r from the origin, being proportional to the chance of survival, is

$$\lambda \exp[-\mu t - r^2/2\sigma^2 t] dt.$$

Then the probability of eggs appearing at a distance r or more from the origin, in the interval $t, t+dt$, is

$$\mu \exp[-\mu t - r^2/2\sigma^2 t] dt.$$

Thus the probability that an egg will be laid at a distance greater than r from the origin is

$$\mu \int_0^{\infty} \exp[-\mu t - r^2/2\sigma^2 t] dt = (r/\sigma) \sqrt{(2\mu)} K_1[(r/\sigma) \sqrt{(2\mu)}],$$

where $K_1(z)$ is the modified Bessel function of the second kind, of order 1 (see for example, Watson 1944).

In this distribution, it is seen that $\sigma/\sqrt{2\mu}$ is a scale factor. The ratio $2\sigma^2/\mu$ is in fact the variance of the distribution of larvae about the central point. Thus, the distribution of the distances r provides information about the ratio $2\sigma^2/\mu$, but not about its components σ^2 and μ . To evaluate σ and μ it is necessary to have information about the distribution and mortality of the moths themselves.

The estimation of the variance of the larval distribution will be considered in a later section. In the following we shall for convenience alter the scale so that the variance is unity, and write the probability as

$$2r K_1(2r). \quad (1)$$

This probability may be differentiated with respect to r , to give the probability density of r :

$$2r \int_0^\infty \exp[-t-r^2/t] dt/t = 4r K_0(2r), \quad (2)$$

which involves the Bessel function of order zero.

The moments of r about zero are found to be

$$\mu_i = \{ \Gamma(\frac{1}{2}i+1) \}^2,*$$

so that

$$\mu_1 = \pi/4 = 0.7854; \quad \mu_2 = 1; \quad \mu_3 = 9\pi/16 = 1.7671; \quad \text{and} \quad \mu_4 = 4.$$

The distribution about zero is leptokurtic, with

$$\delta = 1.$$

The distribution has circular symmetry. A central section of the distribution (corresponding, for instance, to the distribution of one coordinate x , conditional on the other coordinate y being zero), has the density $(2/\pi) K_0(2x)$.

This density is plotted against x in Figure 1. The two-dimensional density is seen to be the solid of revolution produced by rotating this curve about its centre of symmetry. The density has an infinity at zero, corresponding to a high concentration in the neighbourhood of the origin.

The distribution of x is symmetrical. Its even moments are

$$\mu_{2i} = \{ \Gamma(i+\frac{1}{2}) / \Gamma(\frac{1}{2}) \}^2,$$

so that $\mu_2 = \frac{1}{4}$ and $\mu_4 = \frac{9}{16}$. The distribution is markedly leptokurtic, with $\delta = 6$.

Another distribution of practical importance is the marginal distribution of x (the distribution averaged over all values of y). This differs from the conditional distribution, since the coordinates x and y are not distributed independently in the Bessel distribution.

* The symbol μ conventionally represents a mortality rate; usually in other contexts, μ_i represents the i th moment of a distribution; since one is distinguished by a subscript, the use of both should cause no confusion.

The marginal distribution is simply the double exponential:

$$\exp (-2|x|).$$

This distribution, being simple in form, may have some advantages for estimation purposes, where the distribution in two dimensions is adequately determined. (In application to Wildbolz and Baggiolini's data, it was practicable only to fit the distribution in one dimension, that is, the conditional distribution.)

In the practical application of this distribution, the densities in the neighbourhood of the origin will be less than expectation since the numbers of fruits liable to infestation will limit the numbers of larvae found. Nevertheless, as Geier's work shows, the distribution observed is fitted quite well by the Bessel function distribution.

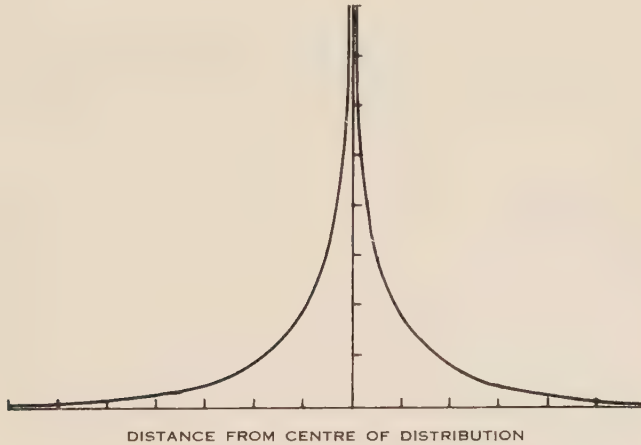


Fig. 1.—Central section of theoretical distribution of larvae.

From the form of the moments it appears that, when $y = 0$, x is distributed like the product of two independent normal deviates; likewise r^2 is distributed like the product of two exponential variables. The general result, of which these are special cases, is that the squared distance in k dimensions is distributed as the product of two independent χ^2 variables, each with k degrees of freedom. These relations provide a convenient means of studying the properties of these distributions.

III. ALTERNATIVE ASSUMPTIONS LEADING TO BESSEL DISTRIBUTIONS

It is a common experience that, although some quantitative or mathematical law can be fitted to a phenomenon, the formulation does not give conclusive evidence on the set of causes to which the phenomenon may be attributed. This is because different cause systems can often lead to the same type of mathematical law. The value of such formulations, however, is that they enable the experimenter to limit the kinds of cause systems that are applicable, and to design critical experiments to decide between them.

In Section II we gave a set of assumptions about the behaviour of the codling moth which led to the distribution discussed there. We now give various other assumptions, by no means exhaustive, leading to the same distribution. That they do lead to this form of distribution will not actually be demonstrated.

All the sets of assumptions have this in common, that they associate with a normal distribution an exponential distribution of one of its parameters; this leads in each case to a distribution of r^2 equivalent to that of a product of independent exponentially distributed variables.

Assumption 2:

- (a) All moths are equally mobile.
- (b) Either the rate of egg-laying, or the number of suitable sites for laying in, decreases exponentially with time, for any cause whatever.

This assumption is a generalization of I. If the suitable sites (i.e. fruits) decrease with time, the sites will tend to be infested more than once, but only one larva will survive on each site. Thus the expected distribution of larvae will be the same as though all were equally viable, and the rate of egg-laying decreased exponentially with time.

Assumption 3:

- (a) The energy (i.e. mean square velocity) of different insects is exponentially distributed.
- (b) Each female is equally fecund, but lays only once, at a fixed time t_0 .

On this assumption, the distribution of *moths* at any time follows the Bessel distribution. Hence, if all eggs are dropped at the one time, the larvae will be distributed in the same way as the moths.

IV. FITTING THE BESSEL DISTRIBUTION

We shall assume that the data conform to one of the three basic distributions given in Section II, and that the centre of the distribution is known, so that only the scale parameter of the appropriate distribution requires to be estimated. With codling moth, the centre is not likely to differ from the point of release; in other applications, since the distribution is strongly peaked, the centre can often be located by inspection.

The problem of estimation is then to determine the spread of the distribution, measured by the mean square value of r ; this is $2\sigma^2/\mu$, which we shall denote by ρ^2 .

When data for the two-dimensional distribution are used, the joint density of a set of n distances r_1, r_2, \dots, r_n is, from (2)

$$(1/\rho^{2n}) (4^n r_1 r_2 \dots r_n) K_0(2r_1/\rho) K_0(2r_2/\rho) \dots K_0(2r_n/\rho).$$

On maximizing this expression with respect to ρ , we find for the maximum-likelihood estimator of ρ the solution ρ_1^* of the equation

$$n\rho_1^* = \sum_i r_i [K_1(2r_i/\rho_1^*)]/[K_0(2r_i/\rho_1^*)].$$

This equation cannot be solved directly, and will need to be solved by iterative methods.

The variance of this estimator is a somewhat complicated function; in large samples, however, its leading term is $0.62\rho^2/n$, about the same as the variance of the simpler estimate given below. The simpler estimator ρ_1 is found by equating the sum of the r_i to its expected value. Then

$$n\rho_1 = 4(r_1 + r_2 + \dots + r_n)/\pi.$$

It is readily verified that

$$\begin{aligned} V(\rho_1) &= [(16/\pi^2) - 1]\rho^2/n \\ &= 0.62 \rho^2/n. \end{aligned}$$

When for any reason values of x , the deviations in one direction alone, are given, ρ will be determined with reduced accuracy. The joint density of a set of n values of x is

$$(1/\rho^n) \exp(-2\sum_i |x_i|/\rho).$$

In this instance, the sum of the absolute deviations is a sufficient statistic for ρ , so that an estimate with all possible accuracy is simply obtained. The maximum-likelihood estimator in this case is

$$\rho_2^* = 2\sum_i |x_i|/n.$$

This is an unbiased estimator of ρ , with variance

$$V(\rho_2^*) = \rho^2/n.$$

Hence the efficiency of this estimator, based on information limited to deviations in one dimension only, is 0.62, or 62%.

Finally we may consider observations taken along a line, as was done by Geier in his analysis of Wildbolz and Baggiolini's data. For this conditional distribution, the density of a sample of n is

$$(2/\pi\rho)^n K_0(2x_1/\rho) K_0(2x_2/\rho) \dots K_0(2x_n/\rho),$$

and the maximum-likelihood estimator ρ_3^* is the solution of the equation

$$n\rho_3^* = 2\sum_i x_i [K_1(2x_i/\rho_3^*)]/[K_0(2x_i/\rho_3^*)].$$

For this estimator the leading term of the variance is $1.47 \rho^2/n$, showing that data from the conditional distribution have only $0.62/1.47$ or 42% of the efficiency of the data on distances in two dimensions.

For this distribution also a simpler estimate is provided by the sum of the absolute values of x . The mean of these is ρ/π and the variance is $[\frac{1}{4} - (1/\pi^2)]\rho^2$ so

that we have

$$\rho_3 = \pi \sum_i |x_i|/n,$$

$$\begin{aligned} V(\rho_3) &= (\tfrac{1}{4}\pi^2 - 1)\rho^2/n \\ &= 1.47\rho^2/n. \end{aligned}$$

The efficiency of this estimator is 42%.

V. ACKNOWLEDGMENT

I am indebted to Dr. P. W. Geier, Division of Entomology, C.S.I.R.O., for proposing this problem to me and for much help in discussion of the biological factors.

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INHERITANCE OF DDT-RESISTANCE INVOLVING THE Y-CHROMOSOME IN THE HOUSEFLY (*MUSCA DOMESTICA* L.)

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Summary

Selection for early maturation applied to a laboratory colony of *Musca domestica* L. eliminated autosomally controlled DDT-resistance from both sexes, but a proportion of the males exhibited a genetically new type of resistance which was shown to be not transmitted through the females but to involve the Y-chromosome. By a single selection with DDT, applied to males only, the early-maturing strain was separated into two true-breeding strains homogeneous in both sexes with respect to DDT-tolerances, the one susceptible to DDT in both males and females, the other susceptible in females but showing at least an eightfold resistance to DDT in all its males.

I. INTRODUCTION

Male insects have, in general, been found to be somewhat more susceptible to toxic substances than females of the same species. Of 59 comparisons between the sexes, reviewed by Busvine (1957), Brown (1958), and Nagasawa (1955), all but two show higher tolerances in the females to stomach or contact poisons. Even when body weight differences have been taken into account, there still remains in many cases a margin of higher resistance in the females. For example, females of *Drosophila melanogaster* Mg. were found to be 1.86 times as resistant as males to DDT applied topically in kerosene solution (Kerr 1954*b*). Correction for body weight reduced the ratio to 1.17 which, however, was still highly significant ($P < 0.01$).

Similar results have been obtained with a laboratory colony of the housefly, *Musca domestica* L., previously described by Kerr *et al.* (1957). In this the ratios of the LD₅₀'s (already corrected for body weight) for females and males ranged from 1.08 to 1.79 for DDT (mean of 22 tests, 1.33), 0.96 to 1.78 for gamma-BHC (mean of 26 tests, 1.34), 1.24 to 1.71 for allethrin (mean of 7 tests, 1.53), and 1.16 to 1.58 for diazinon (mean of 7 tests, 1.37). Two DDT-resistant strains (Kerr *et al.* 1957) derived from this colony by selection with DDT (strain D) or by selection for late maturation (strain L) showed ratios ranging from 1.14 to 2.51 (mean of 28 tests, 1.52) and 1.25 to 2.13 (mean of 6 tests, 1.61) respectively for DDT. Thus the increase in resistance resulting from selection by these two methods accentuated the difference between the sexes. All of the 56 tests with DDT showed higher tolerances in females than in males. In only one of the total of 96 determinations (a BHC test) was the ratio below unity.

This paper describes the selection, from the same colony, of a strain in which the males are approximately 8 times as resistant to DDT as the females, and the investigation of the mechanism of inheritance of this DDT resistance. As outlined

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in a previous brief account (Kerr 1960), the abnormally resistant males were discovered in a strain, designated as E, which was being selected for early maturation (i.e. rapid pre-adult development) by mass-rearing methods in the hope that this would produce a homogeneously DDT-susceptible strain.* Selection was applied to six successive generations by breeding only from eggs laid by flies that emerged during the first 2 days of the emergence period (peak emergence usually occurring on the 4th day). It achieved the desired result in the case of females, which thereafter were homogeneously susceptible for as long as the selection for early maturation was maintained. But in the males there remained a high proportion which survived doses of DDT that killed all females.

II. MATERIALS AND METHODS

All rearing and testing were carried out in an air-conditioned laboratory maintained at $78(\pm 2)^{\circ}\text{F}$. The houseflies were reared by standardized procedures which followed closely those specified in the Peet-Grady method (Anon. 1943).

Puparia were collected from the cultures on the 10th day and, during emergence of the adults, were removed daily to a new cage, thus ensuring that the variation in age was not greater than 24 hr in any cage of test flies. The adults were fed on a 5% suspension of full-cream milk powder in water to which they had access continuously before testing.

Insecticide tests were carried out usually on flies from the maximum-emergence cage when they were 5–6 days old. Twenty flies of one sex were collected in a 4 by 1 in. vial containing carbon dioxide, and while still anaesthetized were weighed as a batch, and each dosed individually on the mesonotum with a volume of insecticide solution proportional to the batch weight. Each batch of 20 treated flies was transferred to a 6 by $1\frac{1}{4}$ in. vial provided with a cotton-wool pad moistened with 10% sucrose solution and closed with a loose cotton-wool plug. Mortalities were determined 24 hr after treatment.

For obtaining dosage-mortality data a geometric series of test solutions of DDT (*pp'*-isomer) in odourless kerosene was prepared. The common ratio of the series was chosen so that not less than five nor more than seven successive concentrations spanned the mortality range. At each concentration the insecticide was applied to at least 40 flies (two batches) of the one sex by means of an optically graduated micropipette (Kerr 1951, 1954a). The volumes applied were of the order of 0.08 and 0.13 μl for males and females respectively. These amounts of kerosene alone were practically harmless. In a series of 26 tests the highest mortality recorded for kerosene-treated males was 5%, that for females 7.5%. Mean mortalities were 1.93 and 2.02%, respectively; thus, on the average, less than 1 fly in 40 could be reckoned as dying from causes other than the insecticide. In many tests the lowest insecticide dosage killed no flies, so that no correction for control mortality was required. When deaths occurred in controls, the observed mortalities for the insecticide dosages were adjusted accordingly by "Abbott's formula". Mortalities

* This was a reasonable expectation because selection in the opposite direction (i.e. for late maturation) had already given rise to a DDT-resistant strain (Kerr *et al.* 1957).

were then transformed to probits (Bliss 1935) and plotted against log dosage. Within the limits of sampling error, the probit values were usually arrayed linearly with respect to log dosage. These transformed data were analysed as outlined by Finney (1952) to give (1) the equation for the best-fitting regression line (hereafter called the *ld-p* line, this being a convenient abbreviation for log dosage-probit line suggested by Hoskins and Gordon (1956)); (2) the variance of the slope of this line; (3) the LD_{50} and its fiducial limits at 95% probability; and (4) the χ^2 value for goodness of fit of the line to the data (which value indicates whether the treated flies were homogeneous or heterogeneous in their individual tolerances).

III. RESULTS

(a) *Composition of Original Colony*

The original unselected colony is characterized by dosage-mortality curves of the type shown in Figure 1. Flattening of the curves in the region of high dosage indicates the presence of abnormally resistant individuals which in this particular generation comprised 17 and 19% of the male and female populations respectively. When these resistant flies are disregarded, the mortalities so adjusted are linearly arranged with respect to log dosage. The lines drawn through them are the *ld-p* lines fitted by the usual maximum-likelihood calculations. In both sexes the departure of the adjusted experimental points from their line is not significant ($\chi^2_{(3)} = 0.97$ and 0.92 for males and females respectively), so that the data strongly indicate the susceptible flies of the population to be homogeneous in their DDT-tolerances. The equations for the lines are:

$$Y = -0.90 + 7.00 (\pm 1.05)x \text{ for males,}$$

and

$$Y = -2.41 + 7.62 (\pm 1.29)x \text{ for females,}$$

where Y = mortality in probits and x = log dosage of DDT (dosage being expressed as $\mu\text{g/g}$ of flies). In slope the lines are not significantly different at the 5% probability level ($\chi^2_{(1)} = 0.14$), and the LD_{50} for females is 1.35 times that for males, a ratio consistent with the mean value of 1.33 for 22 tests with DDT referred to earlier.

Thus the starting point in this selection experiment was the unselected colony as it stood in 1954, consisting, in both sexes, of a mixture of DDT-resistant flies and homogeneously susceptible flies.

(b) *Selection for Early Maturation*

After six generations of selection for early maturation, DDT-resistant females were no longer detected. The relationship between dosage and mortality at this stage is given in Table 1. In females mortality increased to 100% with increasing dosage in a manner which indicated them to be homogeneous in their DDT-tolerances. The males of generation 6, however, were heterogeneous, about 12% of them being resistant to DDT.

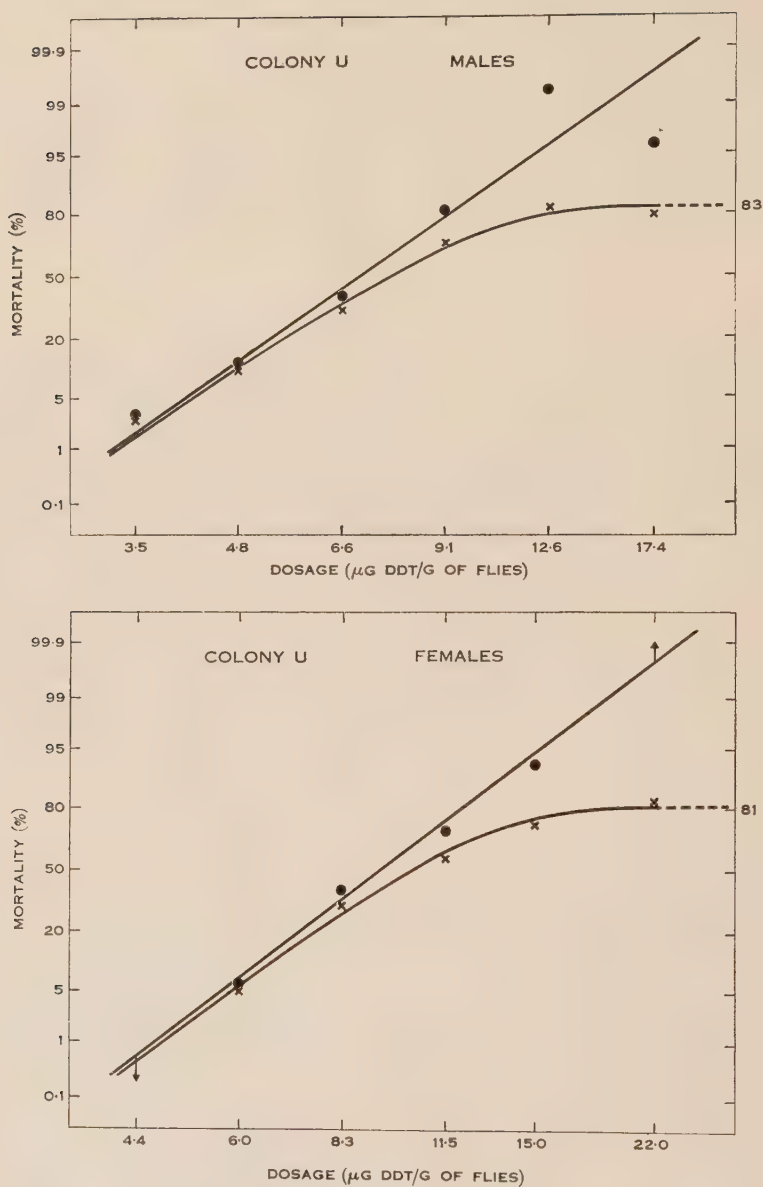


Fig. 1.—Dosage-mortality curves for flies of the unselected colony (U) topically dosed with DDT. The curved lines flattening at 83 and 81% mortality of males and females respectively were fitted to the observed mortalities. The straight lines are the *ld-p* lines calculated for the 83 and 81% of the tested flies that were non-resistant. \times Observed mortality (40 flies per point). \bullet Mortality adjusted for 17% of resistant males or 19% of resistant females. Upward pointing and downward pointing arrows indicate 100 and 0% mortality respectively.

A year later the strain (E) was essentially the same in composition, as shown by the results obtained with generation 23 (Table 2), except that the resistant fraction in the males had increased to about 24%.

TABLE 1
DOSAGE-MORTALITY RELATIONSHIPS FOR FLIES OF STRAIN E GENERATION 6 TREATED WITH DDT
40 flies per dosage

Females*						
Dosage ($\mu\text{g/g}$ of flies)	4.4	6.0	8.3	11.5	15.9	22.0
Mortality (%)	7.5	25.0	50.0	80.0	92.5	100
Males						
Dosage ($\mu\text{g/g}$ of flies)	3.1	4.4	6.0	8.3	11.5	15.9
Mortality (%)	22.5	50.0	72.5	67.5	87.5	87.5

* Regression equation: $Y = 0.10 + 5.38 (\pm 0.56)x$ ($\chi^2_{(4)} = 0.51$), where Y = mortality in probits and x = log dosage of DDT (dosage being expressed as $\mu\text{g/g}$ of flies).

The failure to detect any resistant females when such an obvious proportion of resistant males existed was tentatively ascribed to sampling error and the primary aim to obtain a homogeneously susceptible strain by selection for early maturation

TABLE 2
DOSAGE-MORTALITY RELATIONSHIPS FOR FLIES OF STRAIN E GENERATION 23 TREATED WITH DDT
40 flies per dosage

Females*							
Dosage ($\mu\text{g/g}$ of flies)	2.75	4.05	5.60	7.40	10.55	14.75	20.10
Mortality (%)	2.5	10.0	17.5	37.5	77.5	97.5	100
Males							
Dosage ($\mu\text{g/g}$ of flies)	2.15	2.90	4.15	5.70	8.25	10.20	14.45
Mortality (%)	2.5	7.5	22.5	25.0	60.0	77.5	75.0

* Regression equation: $Y = 0.11 + 5.52 (\pm 0.57)x$ ($\chi^2_{(5)} = 6.04$), where Y = mortality in probits and x = log dosage of DDT (dosage being expressed as $\mu\text{g/g}$ of flies).

was deemed to have been unsuccessful. Selection was discontinued at this stage and no further tests done until generation 46 (Table 3). The reappearance of a small proportion of resistant females and a diminution of the resistant fraction in the males to about 12% seemed to confirm this opinion.

However, the experiment was repeated, with selection for early maturation re-applied from generation 68. Once again after six generations the females showed no indication of resistance (Fig. 2), and the males proved to be a mixture of DDT-resistant and normally susceptible types, this time in the proportion of about 3 : 1.

TABLE 3
DOSAGE-MORTALITY RELATIONSHIPS FOR FLIES OF STRAIN E GENERATION 46 TREATED WITH DDT
40 flies per dosage

Females							
Dosage ($\mu\text{g/g}$ of flies)	2.8	4.0	5.6	8.0	11.2	16.0	22.4
Mortality (%)	2.5	42.5	80.0	95.0	97.5	92.5	97.5
Males							
Dosage ($\mu\text{g/g}$ of flies)	2.0	2.8	4.0	5.6	8.0	11.2	16.0
Mortality (%)	35.0	55.0	82.5	92.5	80.0	95.0	87.5

The part played by cuticular penetration in the resistance of the males was examined at this stage by bypassing the cuticle and injecting DDT in peanut oil into the flight muscles at an average dosage of $20 \mu\text{g}$ DDT/g of flies. Forty males each received $0.12 \mu\text{l}$, and 40 females $0.20 \mu\text{l}$ of the test solution. All the females died,

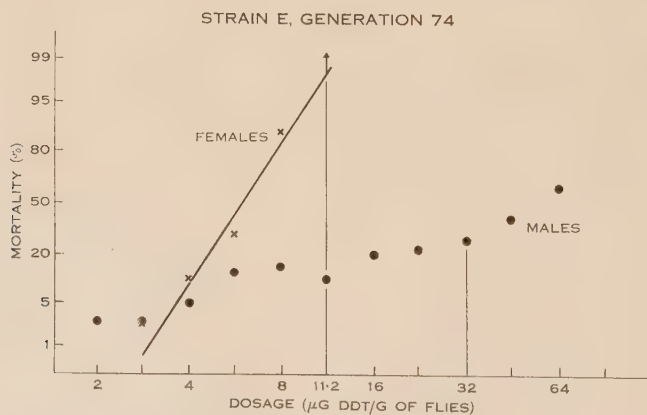


Fig. 2.—Dosage-mortality curves for females and males of strain E generation 74 topically dosed with DDT. \times Observed mortality (40 females per point). \bullet Observed mortality (40 males per point). Upward pointing arrow indicates 100% mortality.

but 85% of the males survived without developing symptoms of DDT poisoning. Thus the resistance of the males was an internal phenomenon and not simply due to exclusion or restriction of entry of DDT by the cuticle.

The distinct flattening of the mortality curve (Fig. 2) for males at about 20% mortality and extending over several dosage increments pointed to the possibility of separating the two types of males by means of selection with differentiating doses of DDT.

(c) *Selection for DDT-resistance and DDT-susceptibility in Males*

In the following generation (75) the sexes were separated before feeding and within 24 hr of emergence, thus ensuring the virginity of the females. The males were treated when 4-6 days old, in batches of 20, at a dosage of 32 μg DDT/g of flies, each male thus receiving almost three times as much DDT (in proportion to body weight) as the amount which had proved lethal to all females of the strain. The total number dosed was 955 of which 727 (76%) survived. About half of these survivors were mated *en masse* with a similar number of the virgin females and a new strain, EY, started with the eggs laid.

A sample of 425 of the resulting adult males (EY generation 1) was tested in the same way. Survival was 95.5%. The mortality of 4.5% indicates that a few of the resistant males having the lowest DDT-tolerances were killed by the test dosage. A dosage lower than 32 μg /g could therefore have been used for selection, but it was necessary to be certain that all non-resistant individuals were eliminated, and the loss of a few resistant males was considered to be unimportant. The test on strain E (Fig. 2) indicated that a dosage of 11.2 μg /g would probably have been sufficient. The strain was then cultured without any further selection with DDT.

In generation 76 of strain E, selection with DDT was applied in the opposite direction to give a strain ES, susceptible in males as well as in females. Sixty-one newly emerged males were collected singly and each one caged with 10 virgin females. Matings were observed to commence on the following day. On the 5th day the male from each cage was tested with DDT at a dosage of 11.2 μg /g. Fifty-two of them survived, and the females with which they had been caged were discarded. It was assumed that the nine males which died were DDT-susceptible, and eggs were pooled and cultured from the nine cages which had contained these males. Samples of the progeny (ES generation 1) were tested with DDT, each fly being weighed and dosed at the rate of 11.2 μg /g body weight. All of 184 females tested were susceptible, but of 174 males tested 3 were surviving at 24 hr without symptoms of DDT toxicosis; they also survived a second dose (13.5 μg /g) and were therefore deemed to be resistant. Thus the first attempt to rid the strain of resistant males in a single selection failed, presumably because one (or more) of the nine male parents was a resistant one that had died during the test from causes other than DDT poisoning, and had thus been classed erroneously among the susceptibles.

In the second attempt (next generation) 32 male parents were classified correctly as DDT-susceptible. None of their progeny in a sample of 600 males and 300 females survived a dose of 11.2 μg DDT/g and similar tests on three successive generations also confirmed the absence of resistant flies from the strain. A dosage-mortality test (Table 4) carried out on generation 7 showed both sexes to be homogeneous in their DDT-tolerances which all lay within a narrow range of dosage below 11.2 μg /g.

On the same day, strain EY was tested in a similar manner for comparison. These results are also given in Table 4. Both sexes were indicated to be homogeneous (χ^2 values not significant) in DDT-tolerances. EY females closely resembled ES flies, their tolerances lying below $11.2 \mu\text{g/g}$. The males of EY all survived a dosage of $11.2 \mu\text{g/g}$, and the slope of their $ld-p$ line was not significantly different from that of ES males, so that the ratio of the LD_{50} 's ($32.28/3.33$) for the males of the two strains may be taken as indicating that EY males were approximately 10 times as resistant

TABLE 4
COMPARISON OF DOSAGE-MORTALITY RELATIONSHIPS FOR FLIES OF STRAIN ES GENERATION 7
AND STRAIN EY GENERATION 8 TREATED WITH DDT
40 flies per dosage

ES females						
Dosage ($\mu\text{g/g}$ of flies)	2.0	2.8	4.0	5.6	8.0	11.2
Mortality (%)	2.5	37.5	75.0	92.5	100	100
LD ₅₀ ($\mu\text{g/g}$ of flies)	3.28 (3.00 and 3.52)*					
Regression equation†	$Y = 1.28 + 7.22 (\pm 0.85)x \quad (\chi^2_{(3)} = 2.47)$					
ES males						
Dosage ($\mu\text{g/g}$ of flies)	2.0	2.8	4.0	5.6	8.0	11.2
Mortality (%)	10.0	32.5	70.0	87.5	100	100
LD ₅₀ ($\mu\text{g/g}$ of flies)	3.33 (2.97 and 3.56)*					
Regression equation†	$Y = 1.94 + 5.87 (\pm 0.70)x \quad (\chi^2_{(3)} = 1.21)$					
EY females						
Dosage ($\mu\text{g/g}$ of flies)	2.0	2.8	4.0	5.6	8.0	11.2
Mortality (%)	2.5	5.0	40.0	85.0	100	100
LD ₅₀ ($\mu\text{g/g}$ of flies)	4.20 (3.91 and 4.54)*					
Regression equation†	$Y = -0.05 + 8.10 (\pm 0.96)x \quad (\chi^2_{(3)} = 6.91)$					
EY males						
Dosage ($\mu\text{g/g}$ of flies)	11.2	16.0	22.4	32.0	44.8	64.0
Mortality (%)	0	10.0	17.5	45.0	75.0	97.5
LD ₅₀ ($\mu\text{g/g}$ of flies)	32.28 (29.44 and 35.39)*					
Regression equation†	$Y = -3.03 + 5.32 (\pm 0.58)x \quad (\chi^2_{(4)} = 3.43)$					

* Fiducial limits at 95% probability.

† Where Y = mortality in probits and x = log dosage of DDT (dosage being expressed as $\mu\text{g/g}$ of flies).

as ES males to DDT applied to the mesonotum in kerosene solution. The slopes of the $ld-p$ lines for EY females and EY males are the only ones in this whole comparison which differ significantly from each other at the 5% level ($\chi^2_{(1)} = 6.2$). Hence no simple value can be derived from this test for the relative resistance of males and females of EY, but comparisons may still be made at specified mortality levels, e.g. the ratio of the LD_{50} 's is approximately 8.

The evidence of these tests strongly suggested that the females played no part in the transmission of this type of DDT-resistance to male progeny. Had they

participated it is difficult to see how the single selections (for resistance and susceptibility) applied only to males could have led successfully to the establishment of the two true-breeding strains EY and ES. The implication was, therefore, that the DDT-resistance of EY males was determined directly by a Y-chromosome factor or indirectly, like sex in some species of *Drosophila*, by the balance between the non-homologous parts of X and Y and the rest of the karyotype. These alternative mechanisms would be rather difficult to differentiate experimentally.

(d) *Genetic Tests*

Since the mechanism of inheritance of DDT-resistance indicated above was unprecedented, further evidence was sought by carrying out reciprocal crosses between the strains EY and ES. The levels of DDT-tolerance in the strains at the starting point of this test are shown in Figure 3. It is clear that the tolerances of males and females of ES and females of EY were all below $11.2 \mu\text{g DDT/g}$, while those of EY males were all above it. This dosage was therefore chosen as the discriminating dose for progeny testing.

(i) *Cross 1: ES Females \times EY Males.*—A sample of 140 females was collected from the maximum-emergence cage of ES generation 7 before feeding and within 24 hr of emergence, thus ensuring virginity. These were caged with 140 males from the corresponding cage of EY generation 8. Eggs were cultured on the 6th day, pupae collected 9 days later, and daily emergence cages of F_1 adults obtained. When 4–6 days old, all normal-sized and apparently healthy males were tested individually with the discriminating dose of DDT. Of 773 tested, 745 (96.4%) survived, a result closely comparable with the 95.5% surviving in the test on EY generation 1. The mortality (3.6%) was considered too small to have any genetic significance. Most of it occurred in flies from the maximum-emergence cage which contained about 1000 flies including females. The mortalities of tested males from the earlier and later cages, which were far less crowded, were 0 and 2% respectively, figures not exceeding the normal expectation for solvent-treated controls. It was considered, therefore, that flies that died in the test had done so from causes other than DDT poisoning.

Obviously the resistance of the F_1 males could not have been transmitted through the female parents since these were from strain ES, the males of which had been exclusively susceptible for 7 generations.

(ii) *Cross 2: EY Females \times ES Males.*—The same procedure as for cross 1 was employed except that 500 virgin EY females were mated with 500 ES males. A total of 1257 F_1 males tested individually with the discriminating dose of DDT all died. These tested males had already mated with the F_1 females which were then used to breed an F_2 generation. The discriminating dose of DDT killed all the 894 F_2 males tested.

The failure of this cross to produce any resistant males in either the F_1 or F_2 generation would convincingly refute any suggestion that EY females were able to transmit either a dominant or a recessive gene for resistance which expressed itself only in males. The results strongly suggested that the EY females did not possess any resistance factor to pass on to their male progeny. However, it was necessary

to demonstrate that the female progeny of cross 2 had neither lost nor gained anything which would prevent them producing male offspring when mated with *Y*-type resistant males, and thus to demonstrate that such a loss or gain was not the reason for none of the male progeny of cross 2 being resistant.

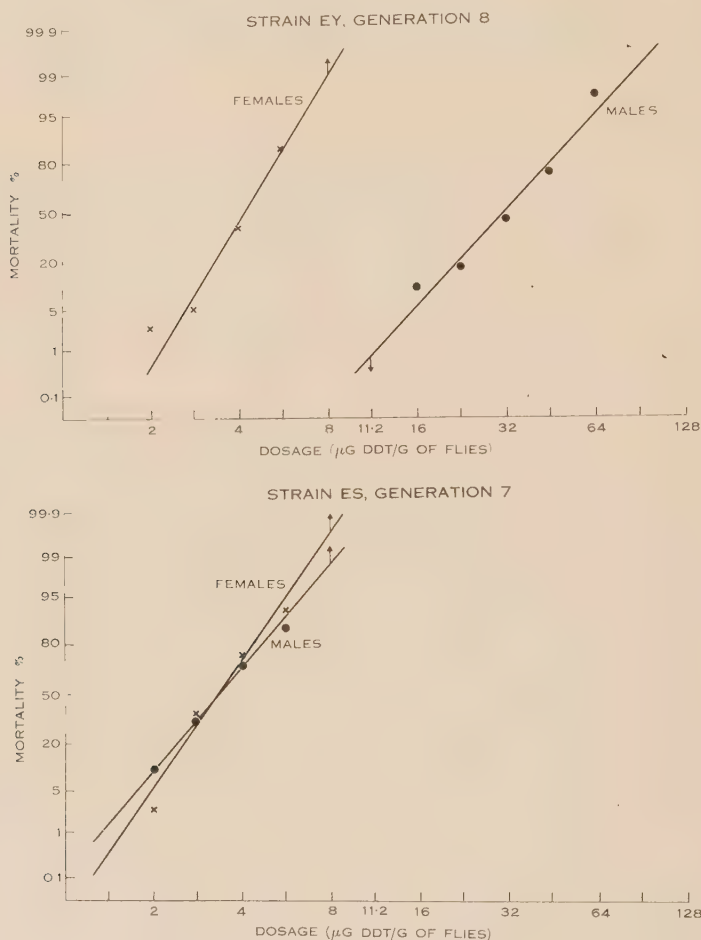


Fig. 3.—Dosage-mortality curves for females and males of strain EY generation 8 and strain ES generation 7 topically dosed with DDT. × Observed mortality (40 females per point). ● Observed mortality (40 males per point). Upward pointing and downward pointing arrows indicate 100 and 0% mortality respectively.

(iii) *Backcross: F₂ Females (cross 2) × EY Males*.—A sample of 500 virgin *F₂* females of cross 2 were caged with 600 EY males of generation 11. After mating, 480 of these EY males were used in a dosage-mortality test to redetermine DDT-tolerance levels which had not been checked for three generations of this strain. Figure 4 shows the experimental points (after adjustment for 5% mortality in the controls) and the calculated *ld-p* line. The departure of the points from the line is

not significant at the 5% level ($\chi^2_{(4)} = 6.75$), so that the data indicate the EY males to be still homogeneous in their DDT-tolerances. The regression equation is

$$Y = -6.28 + 6.99(\pm 0.67)x,$$

where Y and x have the same meanings as previously. The slope of the line is not

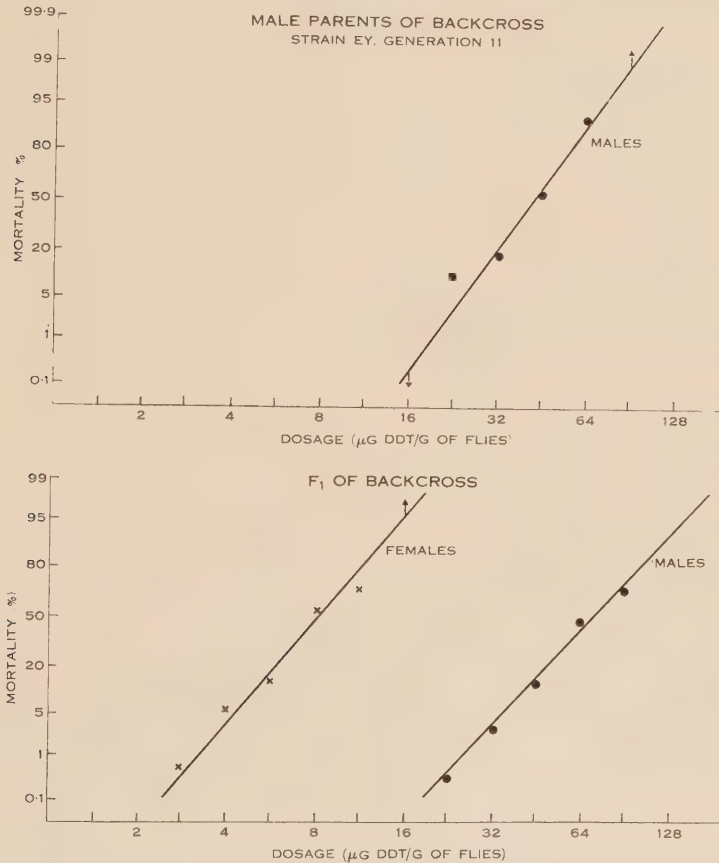


Fig. 4.—Dosage-mortality curves for male parents of backcross (strain EY generation 11) and male and female F_1 progeny topically dosed with DDT. \times Observed mortality after adjustment for mortality in control (40 females per point). \bullet Observed mortality after adjustment for mortality in control (80 males per point). Upward pointing and downward pointing arrows indicate 100 and 0% mortality respectively.

significantly different from that for generation 8. The LD_{50} , $41.1 \mu\text{g/g}$, is somewhat greater than in generation 8 ($32.3 \mu\text{g/g}$), and there seems to have been a general upward shift of tolerances, which, however, does not amount to more than one dosage increment. Thus the actual male EY parents used in the backcross were shown to be the normal Y -type resistant males characteristic of this strain.

Dosage-mortality data were obtained for both female and male F_1 progeny of the backcross. The data after adjustment for 2 and 1% mortality respectively in the controls, are shown in Figure 4 with their $ld-p$ lines, the equations of which were calculated as:

$$Y = -0.33 + 5.82 (\pm 0.70)x \text{ for females } (\chi^2_{(4)} = 5.7),$$

and

$$Y = -4.81 + 5.30 (\pm 0.61)x \text{ for males } (\chi^2_{(3)} = 1.4),$$

where Y and x have the same meanings as previously. The χ^2 values for goodness

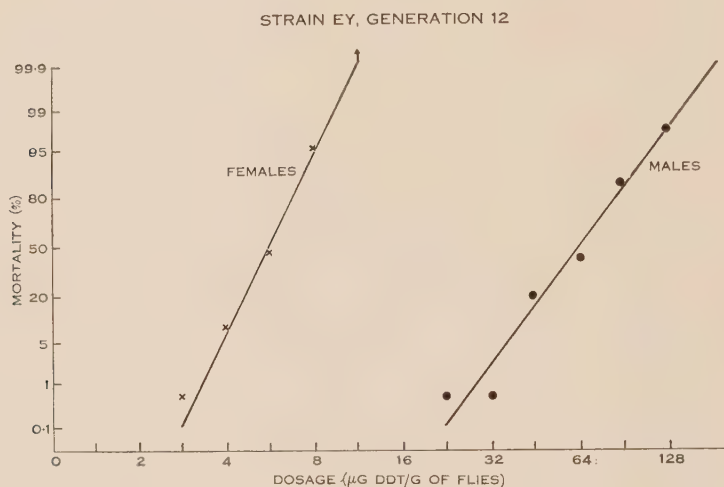


Fig. 5.—Dosage-mortality curves for females and males of strain EY generation 12 topically dosed with DDT. \times Observed mortality after adjustment for 2% mortality in control (40 females per point). \bullet Observed mortality after adjustment for 2% mortality in control (40 males per point). Upward pointing arrow indicates 100% mortality.

of fit indicate both sexes to be homogeneous in tolerances. Since the slopes are not significantly different at the 5% level ($\chi^2_{(1)} = 0.3$), the relative resistance of the sexes is indicated by the ratio of the LD_{50} 's which is 8.6. LD_{50} 's and (in brackets) their fiducial limits at 95% probability are 71.0 (65.0 and 76.5) and 8.25 (7.53 and 9.06) $\mu\text{g/g}$ for males and females respectively. Thus the F_1 males of the backcross were typically Y -type resistants like their male parents, practically none of them being killed by a DDT-dosage just sufficient to kill all their non-resistant sisters. It is interesting to note that although tolerances seem to have undergone a general upward shift during the course of this experiment (cf., for example, the LD_{50} 's above with those for EY generation 8 in Table 4), the Y -type resistant males maintained a more than eightfold resistance as compared with their sisters.

The remainder of the F_1 males of the backcross were divided into two equal groups of 428, one group being treated with the discriminating dose of DDT dissolved

in odourless kerosene as usual, the other group with the kerosene alone. The mortality in the DDT group was 0.93% and in the kerosene alone group 1.87%. Thus the corrected mortality due to DDT was zero, and all the males were therefore DDT-resistant.

The backcross results demonstrate that there was nothing in the female progeny of cross 2 to prevent them reproducing normally when mated with DDT-resistant males. Hence the complete absence of resistant individuals in the F_1 and F_2 generations of cross 2 may be taken as proving conclusively that the females of strain EY do not transmit resistance.

Without exception, the results of the reciprocal-cross experiment verify that the sex-limited DDT-resistance of EY males is determined directly by a Y -chromosome factor or indirectly by the balance between the non-homologous parts of X and Y and the rest of the chromosome set.

(iv) *Stability Check on Strain EY*.—At the conclusion of the genetic test, the DDT-tolerances of strain EY were re-examined. The results (Fig. 5) indicated that both sexes had remained homogeneous ($\chi^2_{(3)} = 0.2$ for females, $\chi^2_{(4)} = 1.8$ for males), and that their tolerance ranges were still completely separated from each other. The equations for the ld - p lines were calculated as:

$$Y = -2.60 + 10.13 (\pm 1.40)x \text{ for females,}$$

and

$$Y = -7.06 + 6.65 (\pm 0.86)x \text{ for males.}$$

In slope the lines were significantly different at the 5% level ($\chi^2_{(1)} = 4.5$), so that, as in the previous test on generation 8 of the strain, no simple value could be derived for the relative resistance of the sexes. However, comparisons could be made at stipulated mortality levels. The LD_{50} 's and their fiducial limits were calculated to be 65.2 (59.5 and 71.0) and 5.62 (5.24 and 6.01) $\mu\text{g/g}$ for males and females respectively. At all mortality levels above 0.1% the resistance ratio for the sexes, i.e. the ratio of equitoxic doses, exceeded 8.

Comparing the results for generations 8 and 12, the regression lines for males are not significantly different in slope ($\chi^2_{(1)} = 1.7$), nor are those for the females ($\chi^2_{(1)} = 1.4$). The LD_{50} for males increased during the four generations by a factor of 2.0, whereas the corresponding factor for females was 1.3. It is not clear why the tolerances of males increased more than those of females. The point to be stressed, however, is that both the susceptible females and the resistant males showed a similar trend in tolerance variation over the period of the genetic test, and this suggests that the upward shift in tolerances shown by the progeny of cross 2 was perhaps no more than the "normal" variation to be expected from generation to generation in these strains.

IV. DISCUSSION

The derivation of the strains selected and the outcome of the genetic tests applied in this investigation are summarized diagrammatically in Figure 6.

The lack of information on the genetic activity of the Y -chromosome in *M. domestica* is a serious handicap to the understanding of the origin of this unique

mechanism of inheritance of DDT-resistance. In the absence of cytological evidence it would appear reasonable tentatively to regard this trait of DDT-resistance confined to certain males as due either to the presence of a new allele at a specific locus on the Y or to a gain (or, less likely, a loss) of chromatic material by the Y. For many genera-

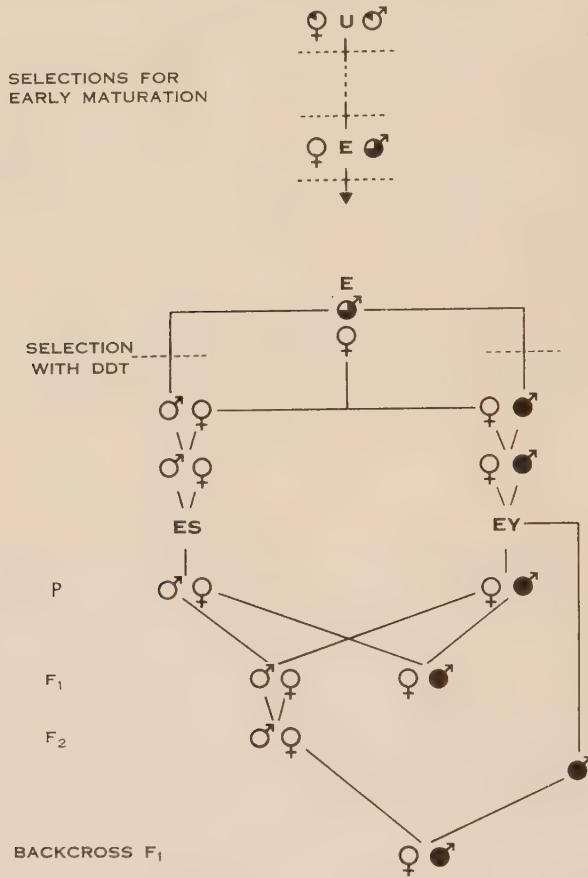


Fig. 6.—Diagram showing the derivation from the unselected laboratory colony of *Musca domestica* of two homogeneous strains, ES (in which both sexes are non-resistant to DDT) and EY (in which the females are non-resistant and the males DDT-resistant), and also the reciprocal cross and backcross tests which proved the holandric inheritance of the DDT-resistance of strain EY males. Sectors of the male and female symbols are blackened to represent the proportions of the populations found to be DDT-resistant. The broken horizontal lines represent selection "screens" through which the flies were "passed".

tions the Y-type DDT-resistant males have coexisted with the normally susceptible males, so that, if a gain or loss of chromatin by the Y were involved, it would need to be such as not to impair fertility.

The Y-chromosome in Diptera has generally been regarded as relatively inert genetically, consisting predominantly of heterochromatin with few or no genes.

However, Tate (1947) found a genetic locus concerned with eye colour on the *Y*-chromosome of *Calliphora erythrocephala*, and there are some genes or at least specific regions in the *Y*-heterochromatin of *D. melanogaster* (see Goldschmidt 1955) and *D. buscki* (Krivshenko 1950) which are concerned with male fertility and development. Bristle size in *D. melanogaster* (Stern 1927) and certain colour traits in the fish *Lebistes reticulatus* (Schmidt 1920) and the beetle *Phytodecta variabilis* (Zulueta 1925) also involve the *Y*-chromosome. Gates (1946) listed 14 abnormal conditions in man for which there is evidence of *Y*-linkage, but Stern (1957) pointed out that the evidence for complete *Y*-linkage was inconclusive in all 17 reported cases in man. These species (excepting *C. erythrocephala*) appear to be the only ones in which holandric inheritance has been observed. Thus the DDT-resistance confined to males in the strain of *M. domestica* described here is thought to be the first record for this species of a major characteristic, outside of sex and fertility, being determined by a *Y*-chromosome factor.

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GROWTH OF THE MOUSE COAT

VIII. CHANGES IN THE COAT AND BODY WEIGHT UNDER HEAT STRESS

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Summary

Observations were made on the hair growth and body weight of mice subjected to high temperature stress. It was found that:

- (1) The critical temperature at which the influence of the high temperature becomes noticeable is about 34°C.
- (2) At 38°C the effect is pronounced, the eruption of adult coat is delayed, and the body weight depressed as compared with controls.
- (3) All hairs were finer and shorter in treated animals.
- (4) The structure of the coat becomes affected in the sense that the number of zigzags is increased and the number of auchenes decreased, as compared with controls.

I. INTRODUCTION

Bonsma (1949) and Turner and Schleger (1960) have shown a close association between hair growth and body growth in cattle. The causal relations between the two are far from clear. Cattle which have a short flat coat increase in body weight at a faster rate, particularly in hot unfavourable environments, than cattle with long silky coats. A short flat coat indicates, at least in hot climates, general thrift and fertility. As mice are more easily handled experimentally than cattle, an exploration in mice of the association between coat and body growth rate in hot environments may help to explain the association in cattle, though the physiology of the two species is different in many respects. This paper describes an investigation into the effect of heat on growth rate in mice and on associated changes in the growth of the coat. This investigation was undertaken prior to an examination of the growth of mice with different types of coat in order to determine the temperature at which effects of heat are marked and the types of changes in coat which take place. These observations may help in classifying mice according to coat type.

The mouse coat is replaced by new hairs at intervals which in early life are rather regular. The first coat is initiated before birth and completes its growth by the time the mouse is 18½ days old (Fraser 1951). This coat is the baby coat and will be referred to as G₁ or the first hair generation. The second coat completely replaces the first and will be referred to as G₂. The replacement is complete insofar as the shafts of the hairs of G₁ lose contact with the follicle bulbs; these grow new hairs

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belonging to G_2 . Both hairs, one G_1 and one G_2 , usually remain in the same hair canal of each follicle, however. Initiation of G_2 is not simultaneous all over the body but starts at the head and in most cases spreads rapidly towards the tail. As G_2 is initiated between 30 and 40 days after birth and as weaning to 40 days is a period during which weight increases rapidly, this period was chosen to test the effect of heat on body and hair growth. The eruption of hairs of G_2 has been described by Dry (1926), Borum (1945), and Fraser and Nay (1955).

The effect of heat on the composition of the coat, the dimensions of the hairs composing it, and the rate of growth of the coat was also observed. Dry (1926) classified the coat into hairs of four kinds: *A*, guard hairs; *B*, awls; *C*, auchenes;

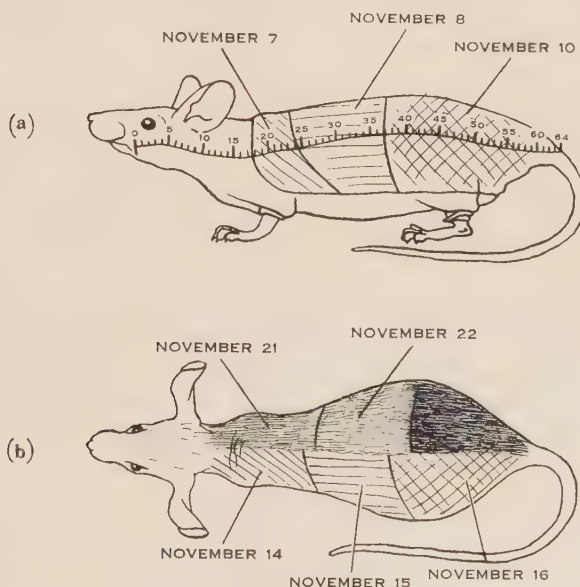


Fig. 1.—(a) Method of recording the eruption of G_2 hairs;
(b) method of recording growth rate of G_2 hairs.

and *D*, zigzags. This classification is followed here, the composition of the coat being expressed as the proportion of hairs of type *B*, *C*, and *D*. *A* has been ignored, as guard hairs form a very small proportion of the total.

II. MATERIAL

Two stocks of mice were used. One is a non-inbred stock of albino mice kept in the laboratory, and which was founded by crossing several different lines. The second stock, also made by crossing several stocks together, and maintained non-inbred, is of recent origin and is segregating for many different colour genes including albino. This stock was used to determine the time taken to complete G_2 . The albino stock was used in all other experiments.

III. EXPERIMENTAL

(a) The Timing of G_2 at 20°C

Forty male and 40 female mice were clipped shortly after weaning, close to the skin on the left side of the body. Mice were weaned at 21 days in this and all subsequent experiments. The eruption of G_2 hairs could be seen clearly on the clipped side and the date at which hairs appear on different areas was recorded on the silhouettes as shown in Figure 1(a). The method is described in more detail by Fraser and Nay (1955). G_2 was considered complete when hairs had appeared on all areas

TABLE 1
AVERAGE AGE (IN DAYS) AT THE ERUPTION OF G_2 IN 40 MALE AND
40 FEMALE CROSSBRED MICE

Position	Males	Females
20	30.92	33.50
30	31.92	37.25
40	32.77	38.17
50	33.30	38.50

of skin. The average age at which G_2 hairs appear in positions 20, 30, 40, and 50 is given in Table 1. It will be seen that in males the process starts on about the 31st day of life and takes about 2.5 days, passing from head to tail. Females start when a few days older and the process takes 5 days to complete. Males and females must, therefore, be considered separately.

TABLE 2
AVERAGE WEANING WEIGHT AND AVERAGE WEIGHT AND AGE AT THE ERUPTION OF G_2 OF MALE
ALBINO MICE KEPT AT 20, 34, AND 38°C FROM TIME OF WEANING

Group	Temp. (°C)	No. of Animals	Average Weaning Weight (g)	Average Weight at Eruption of G_2 (g)	Average Age at Eruption of G_2 (days)
Control	20	19	9.40	17.33	33.31
Hot room	34	19	9.67	16.56	34.78
Control	20	10	8.73	17.37	34.4
Hot room	38	10	9.07	15.10	48.9

(b) Temperature at which Heat Stress becomes Evident

Thirty-eight males from seven litters were divided into two groups. One was kept in the mouse house at 20°C. The other was put into a 1500-egg capacity commercial egg incubator in cages. The temperature was kept at 34°C and the humidity was not controlled. A second lot of 20 males was divided into two groups,

the first being kept at 20°C, and the second being kept in the incubator at 38°C. All mice were weighed at weaning and at intervals thereafter. The completion of G_2 was recorded for both sets of controls and both treated groups. The results are set out in Table 2 and Figure 2. At 34°C the body weight increases are a little less and the completion of G_2 is slightly later than at 20°C. The weights were recorded at weaning and at completion of G_2 so that the treated group was growing a little longer than the control. It seems probable that though the effect of 34°C is not marked there is some retardation of growth. At 38°C the effect was more noticeable. The completion of G_2 took 14 days longer and at 7 weeks of age the control group

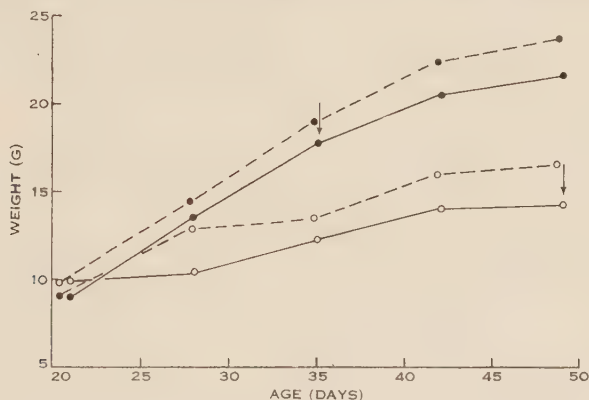


Fig. 2.—Effect of heat stress (38°C) on body weight and the eruption of G_2 hairs (○). Arrows mark the eruption of G_2 over the whole back of the animal. The dotted lines refer to a second experiment in which the eruption of G_2 was not observed. ● Controls.

weighed 22 g whereas the treated group weighed only 14 g. At the stage when G_2 is completed the controls weighed more than the treated even though the treated took 2 weeks longer to reach this stage. It appears, therefore, that 34°C is very near the critical temperature above which growth is seriously interfered with, and at 38°C the effect is more pronounced. Further experiments were carried out at 38°C. The first of these was a repeat of the effect of temperature on growth rate of males. Twenty-two males from four litters were divided into two groups, control and treated, and weighed at weaning and at weekly intervals to 7 weeks. The results are shown graphically in Figure 2 as dotted lines. Both treated and controls were heavier than similar groups in the first run at 38°C but the difference due to treatment was much the same.

(c) Effect of Heat on Hair Growth Rate and on Coat Composition

The third experiment was carried out with 46 mice from nine litters, 23 mice being kept as controls and 23 being kept at 38°C. There were 12 females and 11 males in each group. In addition to being clipped at weaning on the left side, the hair on the right side of these animals was dyed with commercial hair dye. When the hair

of G_2 reaches a length almost the same as G_1 the white hairs of the albino coat can be seen through the dyed G_1 coat (Fig. 1(b)). The time at which the coat changes colour in this way can be recorded to the nearest day. This time was taken as the time required for G_2 to reach a length just short of G_1 and is used as a measure of hair growth rate. The change in coat colour was recorded on silhouettes in the same way as the appearance of G_2 hairs. The difference in days between the two events in each area is taken as an index of hair growth rate. The results are shown in Table 3. The rate of growth of hairs of treated males is slightly less than that of controls, the rate of growth of hairs of females is more than 10% less. The method has limitations, nevertheless it is fair to say that hair growth rate is probably retarded and not accelerated by heat. In treated females the difference in rate of hair growth was significant (at the 5% level) but in treated males it was not significant.

TABLE 3
TIME TAKEN FOR HAIRS OF ALBINO MICE TO REACH A GIVEN LENGTH

Group	Temp. (°C)	Males		Females	
		No. of Animals	No. of Days	No. of Animals	No. of Days
Control	20	11	7.27	12	7.33
Hot room	38	11	7.72	12	8.75

Samples of hair were plucked from all mice in the last experiment and classified into types *B*, *C*, and *D*. Their length and diameter were then measured. One sample was plucked from each mouse at position 40 on the clipped side so all hairs belonged to G_2 . One hundred hairs were picked from the lower end of the sample to avoid discrimination. Only hairs with a club intact were taken to avoid measuring broken hairs. Classification was made in cedar-wood oil under a dissection microscope. Lengths were measured against a graticule and hairs were straightened against the measuring edge. Diameters were measured under a microscope at a magnification of $\times 350$. The broadest part of the *B* hairs, the broadest part of the second segment from the tip of the *C* hairs, and the broadest part of the "stalk" of the *D* hairs were measured. The results, set out in Table 4, show that the composition of the coat differed after heat treatment. The number of *D* hairs was higher and of *C* lower than in the controls. The difference in the number of *D* hairs between treated and non-treated groups was significant at the 1% level, and in the number of *C* hairs at the 0.1% level. There was no significant difference in the number of *B* hairs. All hairs were shorter and finer after treatment. The effect on males and females was not very different. Ten mice were sampled 12 days after the first sample to check that growth had ceased at the time of first sampling. As the measurements

TABLE 4
EFFECT OF TEMPERATURE ON COAT COMPOSITION OF ALBINO MICE

Group	Temp. (°C)	Sex	Hair Generation	Type B			Type C			Type D		
				%	Length (mm)	Dia. (μ)	%	Length (mm)	Dia. (μ)	%	Length (mm)	Dia. (μ)
Control Hot room	20	Male	G ₂	19.63	8.36	48.84	16.80	7.83	27.78	60.27	6.95	19.99
	38	Male	G ₂	18.18	6.54	40.08	10.27	6.32	22.25	68.90	5.81	17.19
Control Hot room	20	Female	G ₂	17.16	7.95	48.25	16.75	7.90	27.99	63.41	7.06	19.99
	38	Female	G ₂	19.41	6.40	41.69	9.66	6.30	22.12	68.33	5.82	16.97
Control	20	Male	G ₁	11.30	7.07	33.65	10.80	6.98	22.84	76.00	6.23	15.00

did not differ they have not been included. The length of the G_2 coat at 38°C was in fact shorter than the G_1 coat. Measurements of G_1 coats of males are shown in Table 4 for comparison.

IV. DISCUSSION

Temperatures over 34°C affect the growth of mice. At 38°C their increase in weight is slow and their hair grows more slowly and to a smaller size than at lower temperatures; the appearance of G_2 is delayed and the percentage of different types of hair is different from that of mice kept at 20°C . Dry (1926) and Fraser (1951) have calculated the percentage of hairs of different types seen in G_1 . Dry found 83% of D and 14% of B and C combined. Fraser, in four different strains of mice, found 69–75% of D , 26–28% of B , and 1–3% of C . The G_1 coats scored in this experiment lie somewhere between Dry's and Fraser's counts. Dry observed that a follicle which produces a certain type of hair in G_1 may produce the same type in G_2 but may produce one of a larger type and only very rarely one of a smaller type. Comparing G_1 to G_2 in this experiment, it appears that retardation of hair growth by heat has resulted in fewer follicles increasing their activity so as to grow type C rather than type D hairs, and about the same number producing type B rather than type C though the B hairs are shorter, not longer, than those in G_1 and the increase in diameter over G_1 is less in the heat than at normal temperatures. The interpretation of hair type being related to follicle activity is in accord with work on sheep (Fraser and Short 1960). It is possible that all changes observed were due to a lowering of follicle activity, in which case time of origin of G_2 which is the most sensitive measure, is the best index of the effect of heat on hair growth in mice.

Rensh (1929) observed that animals living in hot climates were smaller and had shorter coats than their relatives living in temperate climates. If the changes observed in this experiment can be considered adaptive, then the fact that animals in hot climates have similar characters genetically fixed in them may be an example of the way a physiological response becomes fixed by selection as a genetic trait. If mice were selected, some for early and some for late appearance of G_2 , one might expect differences between the two groups in response to a hot environment.

V. ACKNOWLEDGMENTS

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A MATERNAL EFFECT ON VIBRISSA SCORE DUE TO THE TABBY GENE

By B. M. KINDRED*

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Summary

An experiment in selection for the number of secondary vibrissae in the house mouse is being conducted on a stock in which the tabby (*Ta*) gene is segregating. A maternal effect on the vibrissae scores of the progeny of mutant females was observed. This has been investigated in the selection line, stocks derived from it, and *Ta*/inbred backcross stocks.

I. INTRODUCTION

An experiment is being conducted in selection for the number of vibrissae in the minor groups on the face of the mouse (Dun and Fraser 1959; Fraser and Kindred 1960, 1962). As the number of these secondary vibrissae is almost invariant in wild-type mice, tabby (*Ta*), a sex-linked semidominant gene which reduces the vibrissa score from the normal 19 to 10–18 in *Ta*+ females and 5–13 in *Ta*· males or *TaTa* females, was kept segregating in the stock. The mating system was such that *Ta*+ females were used as parents one generation and ++ females in the next. The response to selection was quite satisfactory but it soon became apparent that *Ta*+ females in the low line were giving a peculiar zigzag response and examination of the high line revealed a similar but much less pronounced phenomenon (Fig. 1).

With almost complete regularity *Ta*+ progeny of *Ta*+ females and ++ males had higher scores than the progeny of ++ females and *Ta*· males. There are several possible explanations for such a response:

- (1) Different selection pressures when selecting on *Ta*+ in one generation and on both *Ta*+ and *Ta*· in the next.
- (2) Different factors involved in determining the vibrissa score of *Ta*+ females and *Ta*· males so that selection is not for the same thing in each generation.
- (3) A maternal effect such that *Ta*+ females mated to ++ males produce *Ta*+ offspring which are closer to normal than those produced by ++ females mated to *Ta*· males; i.e. the offspring tend to resemble the male parent.
- (4) A paternal effect—although the possibility of paternal effects is usually disregarded since the cytoplasmic contribution of the male parent is so small, it should be considered as the phenotype of the *Ta*+ offspring approaches that of the male parent, not the female.
- (5) Some uncontrolled environmental factor which affects the vibrissae and which happens to vary in such a way as to affect alternate generations.

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II. MATERIALS AND METHODS

The basic mouse stock for this work was Fraser and Dun's tabby selection stock (HST, LST) which was originally formed by crossing the CBA and 101 inbred lines with three stocks containing the *Ta* gene (Dun and Fraser 1959). During the first few generations of selection there was some separation so that the high line was composed predominantly of 101 and one tabby stock, while the low line was mainly CBA and another tabby stock. The original plan was to mate *Ta*+ females and +· males—this would produce *Ta*+ females, ++ females, *Ta*· males, and +· males each generation, allowing comparison at three levels of expression, hemi-

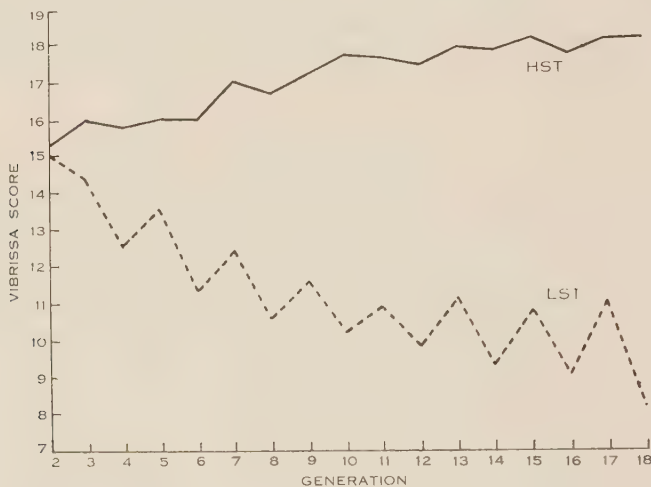


Fig. 1.—Response of vibrissa score to selection in HST and LST *Ta*+ females.

zygous, homozygous, and non-tabby. After the second generation the system of mating *Ta*+ females and +· males one generation and ++ females and *Ta*· males the next was adopted to increase the selection differential.

Selection was only on tabby mice, and no selection on normal mice was ever practised. The method of selection was to rank all litters according to the mean vibrissa score of the *Ta*+ and *Ta*· mice; ++ and +· mice were selected on litter ranking alone, *Ta*+ and *Ta*· were selected on their own score but where two or more mice had the same score the litter ranking was used. Matings were assortative, i.e. the best females were mated to the best males except that sib-matings were avoided.

Relaxed selection lines (XHST, XLST) were derived from the 12th generation of HST and LST. In these lines mating was at random but the mating pattern of the tabby selection (ST) experiment was retained. The maternal-effect lines (HME, LME) were established from the 13th generation of ST. Selection was carried out in exactly the same way as for ST but *Ta*+ females and +· males were mated each generation. Other stocks used were made by crossing the tabby gene into A, CBA, C57, 101, and DBA inbred backgrounds (*Ta*/A, *Ta*/CBA, *Ta*/C57, *Ta*/101, and *Ta*/DBA). Six gene-

rations of backcrossing were completed. These stocks were not selected on vibrissae but the ST system of mating was used except in *Ta*/A where after three generations of backcrossing to A strain, the *Ta*· males were too inviable to use for matings.

Small and weak *Ta*· males also occurred in *Ta*/DBA and to a lesser extent in *Ta*/101 and *Ta*/C57. These were not used for mating. It has been found (Fraser and Kindred 1960) that vibrissa score shows little, if any, correlation with viability so this selection should not affect the vibrissa scores.

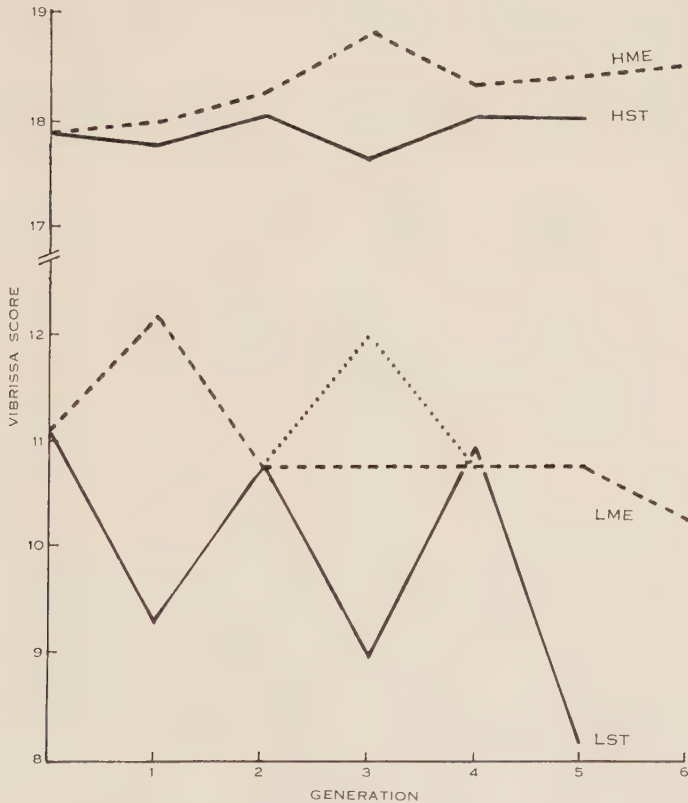


Fig. 2.—Comparison of mean vibrissa scores of *Ta*+ females in tabby selection and maternal-effect lines.

Scoring in all lines was done at 5 days and checked at 10 days of age when the coat colour and tabby genotype were also recorded. All scoring was done under a desk lamp with a dark background. Vibrissa which were less than half the normal length were not scored.

III. RESULTS

To show that the zigzag response was due to the mating system and not some uncontrolled variable, the maternal-effect selection lines were set up. From HST and LST generation 13 ++ females and *Ta*· males were selected as usual for gener-

ation 14 matings but an equal number of $Ta+$ females and $+$ males were selected in exactly the same way to begin HME and LME. Figure 2 shows the mean vibrissa scores of $Ta+$ females from these lines compared with subsequent generations of HST and LST.

In the first generation, as expected, the offspring of $Ta+$ females had higher scores. LME mice average 2-4 more vibrissae than LST. The high line as usual shows much less effect but HME is 0.2 vibrissae higher than HST. Considering first the high line there was a steady increase in vibrissa score except for one generation. No sign of zigzagging appeared at all though HST over this period showed small but regular fluctuations. In LST the zigzags are very pronounced. After the first generation rise, LME scores dropped under the influence of selection but in subsequent generations there were insufficient $Ta+$ females for effective selection to be practised. It was expected that this would cause the line to flatten, and this

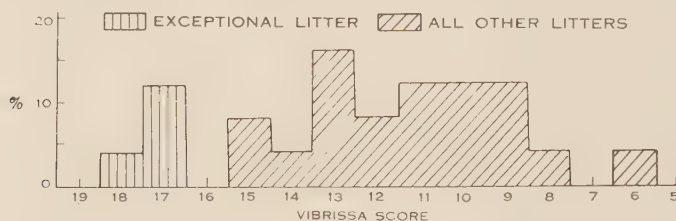


Fig. 3.—Distribution of vibrissa scores of $Ta+$ females in LME, generation 3.

was the case except in generation 3 where there was again an increase (shown in Fig. 2 as a dotted line). This looked startlingly like a reverse zigzag but examination of the data showed that the increase was entirely due to one litter consisting only of $Ta+$ females with very high scores. A frequency distribution (Fig. 3) showed this litter to be exceptional and analysis of variance for all generations showed highly significant heterogeneity in generation 3 but none in other generations or in generation 3 without this litter (Table 1). In view of this heterogeneity and the fact that descendants of this litter in test matings showed abnormal segregation ratios and very high scores, the litter was omitted from the results.

The elimination of the alternating response when $Ta+$ females were used every generation demonstrates clearly that the zigzag effect is due to the mating system and not to external environment.

Comparison of HST and LST with the lines on which selection was suspended (XHST and XLST) will demonstrate whether the first and second possibilities, both of which involve differences in selection could be responsible. Figure 4 shows mean scores of XHST and XLST $Ta+$ females plotted with mean scores of HST and LST $Ta+$ females of comparable generations. The zigzags in the random-mated high line are, if anything, more pronounced than in the selected line and in the two low lines the responses are almost identical. The effect, therefore, cannot be the result of qualitative or quantitative selection.

To test the possibility of a paternal effect, $Ta+$ and $++$ sibs from HST and LST were mated to the same $Ta+$ males. If the difference in response is due to the males, $Ta+$ females from these matings should not differ in vibrissa score but if the females are responsible, offspring of $Ta+$ females should still have higher scores than offspring of $++$ females. In the high line the mean score of $Ta+$ offspring of $Ta+$ females was 18.2 and that of $Ta+$ offspring of $++$ females was 18.1.

TABLE 1
ANALYSIS OF VARIANCE FOR EACH GENERATION OF LOW MATERNAL-EFFECT LINES

Generation	Source of Variation	D.F.	S.S.	M.S.	<i>F</i>
1	Mean	1	4176		
	Between litter	10	53.46	5.35	0.629
	Within litter	9	76.54	8.50	
2	Mean	1	2937.6		
	Between litter	9	101.9	11.32	2.551
	Within litter	8	35.5	4.44	
3	Mean	1	3552.2		
	Between litter	8	247.05	30.88	6.599*
	Within litter	7	32.75	4.68	
4	Mean	1	3920.1		
	Between litter	15	114.0	7.60	1.807
	Within litter	14	58.9	4.21	
5	Mean	1	2552.49		
	Between litter	11	87.91	7.99	1.644
	Within litter	10	48.6	4.86	
6	Mean	1	2421.36		
	Between litter	12	86.24	7.19	1.780
	Within litter	11	44.4	4.04	
3†	Mean	1	2496.1		
	Between litter	5	112.9	22.58	2.823
	Within litter	4	32	8.00	

* Significant at 1% level. No other *F* values are significant.

† Without odd litter and related litters.

The corresponding scores for the low line were 9.9 and 8.2. The difference between LST and LME is significant, that between HST and HME is not. These differences, however, compare satisfactorily with the differences found in successive generations in HST and LST, so it is clear that $Ta+$ mothers produce progeny with higher scores than $++$ mothers, i.e. a maternal effect is operating.

It is not possible to distinguish between the effects of intra-uterine environment and conditioning of the egg cytoplasm without performing transplantations of ova. This is a very difficult technique and requires more time, mice, and space than were available. It is, however, comparatively simple to test for a post-partum maternal influence by exchanging litters between females of different genotypes at birth. There is little likelihood of this occurring with vibrissae of $Ta+$ mice because usually all vibrissae are present at birth (Dun 1959). As expected, it was not possible to alter the vibrissa score after birth even when litters were exchanged between high- and low-line mice.

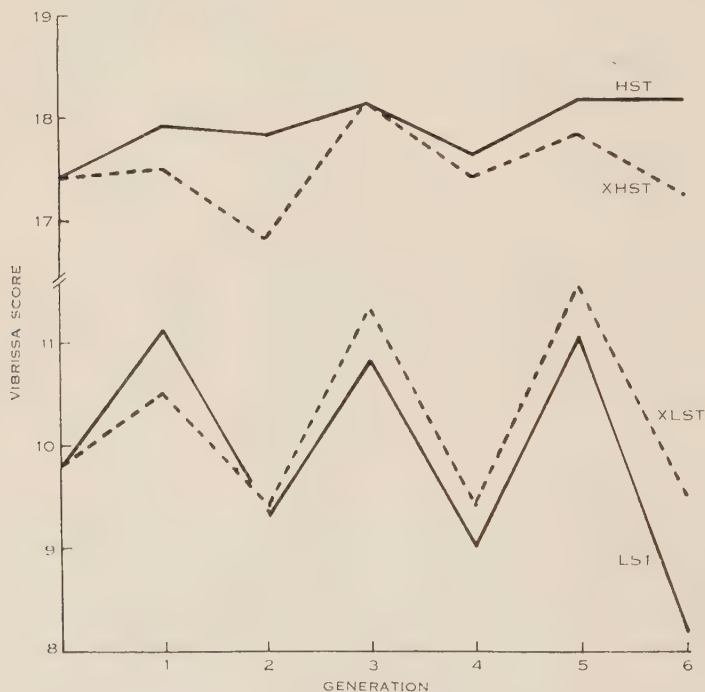


Fig. 4.—Mean vibrissa scores of $Ta+$ females in tabby selection lines (HST, LST) compared with relaxed tabby selection lines (XHST, XLST).

Further information about the nature of the maternal effect can be obtained from the Ta /inbred stocks. Figure 5 shows the mean vibrissa scores for $Ta+$ females of the inbred backcross stocks. The first two generations were not considered as it has already been shown that in an F_1 between two stocks $Ta+$ mice tend to have a higher score than in the parent stock (Fraser, Nay, and Kindred 1959).

In generations 2–6, C57 and CBA showed a marked zigzag; A strain began to give a similar response to CBA until the use of $Ta-$ males had to be discontinued. 101 and DBA produced smaller fluctuations which in 101 were less regular than in the other inbreds. The most striking result is that the response in C57 is in the opposite direction to the response in all the other lines, i.e. offspring of $++$ females had higher scores than those of $Ta+$ females. This, combined with the characteristic

range of the zigzags in the other Ta /inbred lines suggests that the type of response is under genotypic control.

The differences in response in HST and LST are probably due to the predominance of 101 and CBA respectively in their makeup. The characteristic response of 101 is weak and somewhat irregular while that of CBA is regular and marked.

$TaTa$ females provide a third maternal environment from which $Ta+$ mice can be produced. There is, however, no difference in the maternal effect of $TaTa$ and $Ta+$ mothers. When these were compared the $Ta+$ offspring of $TaTa$ had a mean score of 6.9 and those of $Ta+$ a mean score of 6.8. This difference was not

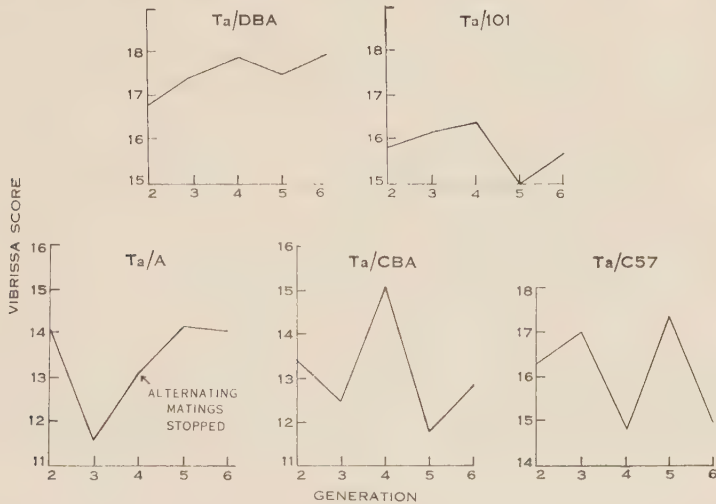


Fig. 5.—Mean vibrissa scores of $Ta+$ females of the Ta /inbred backcross stocks.

significant. In these mice the ulnar-carpal vibrissae were not scored so that the vibrissa scores are markedly lower than those of other $Ta+$ mice discussed here. It was possible to compare the scores of $Ta+$ males from $Ta+$ and $TaTa$ mothers (it is, of course, impossible to obtain $Ta+$ males from $++$ males to study the maternal effect on the more extreme phenotype). Again there was no difference between $TaTa$ and $Ta+$ mothers, the mean score of $Ta+$ males from both groups was 6.3.

IV. DISCUSSION

(a) Cytoplasm or Uterine Environment?

A definite answer to the question of whether the maternal effects on vibrissa score are mediated through the uterine environment or egg cytoplasm cannot be given. The only example of a maternal effect, having no obvious relation to nutrition level, for which the question of cytoplasm or intra-uterine environment has been answered in the mouse, is that of the number of lumbar vertebrae, shown by McLaren and Mitchie (1958) to be due to an intra-uterine effect. There are some indications that this case is similar. Litter size, age, and parity of the mother have no effect on vibrissa score (Tables 2 and 3).

TaTa females are smaller and less viable than *Ta+* females and produce smaller litters. On gross external morphology there is a greater difference between *TaTa* and *Ta+* than there is between *Ta+* and *++*; yet in their effect on the vibrissae of their offspring *TaTa* mice do not differ from *Ta+*. It seems, therefore, that the effect cannot be mechanical or due to nutrition or maternal physiology.

TABLE 2
MEAN VIBRISSA SCORES OF SUCCESSIVE LITTERS IN GENERATION 2

Litter No.	High-selection Line		Low-selection Line	
	<i>Ta+</i>	<i>Ta.</i>	<i>Ta+</i>	<i>Ta.</i>
1	15.5	9.3	15.0	8.7
2	15.0	9.2	15.0	8.9
3	15.5	9.0	15.1	8.9
4	15.1	8.8	15.1	8.1

* Generation 2 was the only generation where several litters were bred.

A drastic effect on intra-uterine environment can be produced by X-rays. Fraser and Hall (1958) found that irradiation of pregnant females produced a reduction in vibrissa score only if given between 8 and 14 days gestation. Irradiation before this time produced no effect whatever. Histological examination of foetuses

TABLE 3
MEAN VIBRISSA SCORES OF *Ta+* MICE FROM LARGE AND SMALL LITTERS IN GENERATION 10*

Litter Size	High-selection Line	Low-selection Line
5—	17.8	10.1
9+	18.4	9.8

* This generation was used because adequate numbers of large and small litters were available.

reveals no evidence of vibrissa formation before 12 days so it seems more likely that maternal effect is produced by some factor which is present between 8 and 14 days rather than by an effect on the egg cytoplasm which would be most effective long before the vibrissae appear and before they can be affected by X-rays. This treatment has not yet been given to *Ta+* foetuses which one would expect to be more sensitive to changes in the environment.

Inbred animals are particularly sensitive to environmental variation (Robertson and Reeve 1952; McLaren and Mitchie 1954). When the *Ta* gene was crossed to inbred strains the maternal effect was more marked than in the less inbred selection lines. This also suggests that the maternal effect is produced by some environmental component. This evidence indicates that these maternal effects are produced by the uterine environment. As mutant females are involved in both it may be that the early growth rate is slightly slower, thus prolonging the period over which vibrissa initiation can take place.

(b) *Genotypic Control*

Most maternal effects which have been reported are matroclinous but most of these can be attributed generally to nutrition. Almost all the others are associated with the skeleton, particularly the skull, and would be subject to the same processes of ossification and skull allometry. Patroclinous maternal effects have been described by Reed (1937), Deol (1955), and Green and Green (1959). The *Ta*+ maternal effect reported here may be either matroclinous or patroclinous. In most of the stocks a patroclinous effect is found but backcrossing to C57 produces a matroclinous effect. The magnitude and regularity of this effect preclude the possibility that this reversal is due to chance; it can only be due to the C57 genotype. Thus it appears that the direction of a maternal effect depends on the genotype of the individuals involved. This is essentially similar to work which has been done on the number of lumbar vertebrae in the mouse. Green (1951) made reciprocal crosses between C57 and BALB/c. The difference between the reciprocal F_1 's was very small but tended to be matroclinous. Reciprocal crosses between C57 and C3H (Green and Russell 1951) resulted in a clear maternal effect also of a matroclinous type yet C57 \times DBA crosses (Green and Green 1959) showed an equally clear patroclinous maternal effect.

Sensitivity to a maternal effect is also genetically determined. *Ta*+ mice show a marked maternal effect while ++ or +· sibs are entirely unaffected. Differences in sensitivity of males and females in the response of lumbar vertebrae to maternal environment also occur. Green and Russell (1951) found in the C57 \times C3H crosses that the response of males was more marked while the reverse was found by Green and Green (1959) in C57 \times DBA crosses. Further differences in sensitivity are found in the *Ta*/inbred backcrosses. The response to the maternal effect had characteristic range in each inbred line. Secondary vibrissae of *Ta*+ mice provide particularly suitable material for the study of maternal effects because of their sensitivity and because the character, vibrissa score, is naturally subdivided into 19 units allowing considerable gradation of objective scoring.

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THE VIABILITY OF FOWL SPERMATOZOA IN DILUTE SUSPENSION

By R. G. WALES* and I. G. WHITE*

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Summary

The motility of fowl spermatozoa is depressed at relatively high dilutions and the use of diluents with a high tonicity is important in helping to offset the detrimental effect.

Protection by as little as 2.5% of fowl seminal plasma is due to a heat-stable substance which is not present in dialysed plasma or in the supernatant after ethanol precipitation.

Supernatants obtained from washed fowl spermatozoa depress motility.

Bovine albumin, bovine globulin, casein, and acacia gum give some protection against the harmful effect of dilution but none of these substances is as beneficial as seminal plasma.

I. INTRODUCTION

The inactivation of mammalian spermatozoa on high dilution has been extensively studied since Milovanov (1934) noted that 1% sodium chloride had this effect. Emmens and Swyer (1948), investigating the motility of rabbit spermatozoa in dilute suspension, demonstrated that the harmful effect of dilution was not due, as Milovanov thought, to the toxicity of sodium chloride, but occurred as readily in sulphate and tartrate diluents. Other investigators have reported similar findings for bull, ram, and human spermatozoa (Salisbury *et al.* 1943; Kennedy 1947; Cheng, Casida, and Barrett 1949; Blackshaw 1953; White 1954).

The viability of dilute rabbit spermatozoa is improved by adding accessory secretion. Supernatants from spermatozoa left overnight, however, are even more effective (Emmens and Swyer 1948). Thus it would seem that the detrimental effect of dilution, in the rabbit at least, is due to both dilution of plasma material and the loss of substances from the spermatozoa.

The nature of the substance or substances lost from spermatozoa at low cell concentration, however, is unknown. White (1953*a*, 1953*b*, 1953*c*) demonstrated that potassium increased the motility and glycolysis of repeatedly washed or moderately diluted ram and bull spermatozoa. At lower cell concentration, however, some other factor must be limiting as potassium is ineffective.

Munro (1938*a*), Bonnier and Trulsson (1939), and Wilcox (1958) have reported lowered fertility with diluted fowl semen, which is partly offset by adding seminal plasma (Munro 1938*b*). Except for the works of Grodzinski and Marchlewski (1935) and Lorenz and Tyler (1951) there is little information on the effects of high dilution on avian spermatozoa *in vitro*, and the studies reported in this paper were, therefore, undertaken.

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II. MATERIALS AND METHODS

(a) Semen

Semen was collected by abdominal massage (Burrows and Quinn 1939). Duplicate spermatozoal counts were made with the neat semen which was then diluted to the required cell concentrations. The spermatozoa were kept at 20–25°C and motility scored at hourly intervals for 4 hr by the system of Emmens (1947). Full motility was rated as four and complete immotility as zero. As quarter grades were frequently used, the actual scores have been multiplied by four, and the total score multiplied by four over the experimental period, the motility index, has been used as unit observation in the analyses of variance.

(b) Diluents

A.R. chemicals and glass-distilled water were used. Relative tonicity (100% = 0.9% NaCl) was calculated on the assumption that there was complete primary dissociation of all electrolytes and no secondary dissociation. The diluent (relative tonicity 140%, pH 7) used in most fowl experiments had the following composition: 0.0136M $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.0064M $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 0.005M KCl, 0.0015M $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.0641M NaCl, 0.25M glucose.

(c) Preparation of Seminal Plasma Extracts

The following extracts were prepared from pooled ejaculates:

(i) *Heated extract*.—Plasma was heated in a boiling water-bath for 10 min, cooled, centrifuged, and the supernatant removed.

(ii) *Ethanol extract*.—One volume of plasma was precipitated with six volumes of redistilled ethanol. The supernatant after centrifuging was evaporated to dryness in an incubator at 37°C and made up to the original volume with glass-distilled water.

(iii) *Dialysed extract*.—The plasma sample was dialysed against glass-distilled water for 2 days at 5°C using "Cellophane" as a membrane.

(d) Preparation of Supernatants

Spermatozoa were separated from accessory secretion by centrifuging at 1500 r.p.m. (approx. 300 *g*) for 15 min, washed once, and made up to the original volume with diluent. Half of the resulting washed suspension was incubated at 37°C for 6 hr and the other half repeatedly frozen and thawed during the same period. The samples were then centrifuged and the supernatants removed.

(e) Chemicals

The vitamins and other fine chemicals were obtained from the following sources: folic acid—Roche Products Ltd.; *p*-aminobenzoic acid, riboflavin, pyridoxine, and niacin—Andrews Laboratories; calcium pantothenate and thiamine hydrochloride—General Biochemicals Inc.; vitamin B₁₂—Merck and Co. Inc.; biotin—Delta Chemical Works; inositol—Difco Laboratories; ascorbic acid, serine, and alanine—British Drug Houses Ltd.; glycine—May and Baker Ltd.; creatine—Light and Co.

TABLE 1

EFFECT OF 0.005M POTASSIUM AND 0.0015M MAGNESIUM ON THE MOTILITY OF FOWL SPERMATOOZA

Mean motility indices for six ejaculates over 4 hr are given

Sperm Density (millions/ml)	Ion Added	Relative Tonicity (0.9% NaCl = 100)		
		100	125	150
200.0	Nil	49.5	55.2	52.3
	K ⁺	47.7	52.2	55.5
	Mg ⁺⁺	48.7	53.2	54.7
	K ⁺ +Mg ⁺⁺	44.0	53.3	56.0
20.0	Nil	6.5	19.8	23.5
	K ⁺	12.2	30.7	38.3
	Mg ⁺⁺	7.2	16.8	28.8
	K ⁺ +Mg ⁺⁺	13.5	34.3	40.3
2.0	Nil	0.7	2.8	5.8
	K ⁺	0.3	5.2	8.3
	Mg ⁺⁺	1.5	3.7	8.0
	K ⁺ +Mg ⁺⁺	0.0	2.2	11.0
0.2	Nil	0.0	0.0	0.7
	K ⁺	0.0	0.8	0.7
	Mg ⁺⁺	0.0	0.2	0.2
	K ⁺ +Mg ⁺⁺	0.0	0.2	0.7

Summary of the Analysis of Variance†

Source of Variation	Degrees of Freedom	Variance Ratio	Source of Variation	Degrees of Freedom	Variance Ratio
Effect of dilution	2	2748.7**	First-order interactions		
Effect of tonicity	2	193.4**	Dilution × tonicity	4	35.3**
Effect of potassium	1	48.4**	Dilution × potassium	2	49.8**
Effect of magnesium	1	1.3	Dilution × magnesium	2	1.3
Ejaculate differences	5	26.8**	Tonicity × potassium	2	9.2**
			Tonicity × magnesium	2	3.2*
			Potassium × magnesium	1	0.0
			Ejaculate interactions	30	4.9**
			Residual	161	15.2

* $P < 0.05$.** $P < 0.01$.

† The results at a spermatozoal concentration of 0.2 million/ml have been omitted from the analysis.

Ltd.; cytochrome *c* and flavine adenine dinucleotide—Nutritional Biochemicals Corp; crystalline bovine albumin and crystalline bovine γ -globulin—Armour Laboratories; crystalline egg albumin—prepared by the method of Cole (1933).

(f) *Statistical Analysis*

The significance of the results has been assessed by analyses of variance which are presented in summary form giving only degrees of freedom and variance ratios for each source of variation.

Where numbers of independent treatments have been compared with controls, the standard error of the difference between each treatment and the control mean has been calculated from the interaction mean square of the analyses of variance,

TABLE 2
EFFECT OF SEMINAL PLASMA ON THE MOTILITY INDICES OF DILUTED FOWL
SPERMATOZOA

Mean values for six ejaculates are given

Sperm Density (millions/ml)	Seminal Plasma Concn. (%)			
	0	2.5	5.0	10.0
20	42	46	43	42
2	11	37	38	37

and the significance of the difference has then been assessed in a *t*-test using the degrees of freedom associated with the interaction mean square.

In cases where motility falls rapidly, variances are probably not completely independent of the level of response. In such instances, however, there is rarely any doubt about the significance of the effects observed.

III. RESULTS

(a) *Effect of Composition of Diluent on Diluted Fowl Spermatozoa*

Potassium (0.005M) and magnesium (0.0015M) chlorides were added alone and in combination to diluents composed of 0.0136M $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.0064M $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 0.0641M NaCl, and enough glucose to bring the relative tonicity to 100, 125, and 150 respectively. Fowl semen was diluted to give final cell concentrations of 200, 20, 2, and 0.2 millions/ml in these diluents and the mean motility scores for six ejaculates are given in Table 1. Motility indices were very low at a cell concentration of 0.2 million/ml and to avoid heterogeneous variances, these results were omitted from the analysis of variance which is summarized in Table 1. Motility at low spermatozoal concentration was maintained best in potassium-containing diluents of relative tonicity 150. Magnesium, on the other hand, had no

effect on diluted fowl spermatozoa. In all subsequent tests optimal conditions were maintained by using the hypertonic diluent containing potassium and magnesium ions.

(b) *Effect of Seminal Plasma*

As little as 2.5% seminal plasma almost completely protected fowl spermatozoa against the harmful effect of dilution and was as effective as higher concentrations of plasma (Table 2). The results of further tests on the effect of seminal plasma are

TABLE 3
EFFECT OF SEMINAL PLASMA ON THE MOTILITY OF FOWL SPERMATOZOA AT 15 AND 25°C

Mean motility indices for duplicate observations on four ejaculates are given

Cell Concentration (millions/ml)	Control		5% Seminal Plasma	
	15°C	25°C	15°C	25°C
200.0	56.3	57.4	54.9	57.9
20.0	55.2	53.7	54.5	56.1
2.0	51.4	46.0	54.4	54.6
0.2	34.4	19.5	51.9	52.8

Summary of the Analysis of Variance

Source of Variation	Degrees of Freedom	Variance Ratio
Between temperatures	1	6.1*
Effect of plasma	1	103.2**
Effect of dilution	3	98.4**
Ejaculate differences	3	3.6*
First-order interactions		
Temperature × plasma	1	17.6**
Temperature × dilution	3	6.7**
Plasma × dilution	3	58.4**
Ejaculate interactions	15	3.3**
Higher-order interactions	64	1.2
Between duplicates	64	19

* $P < 0.05$. ** $P < 0.01$.

shown in Table 3. Fowl spermatozoa diluted to concentrations of 200, 20, 2, and 0.2 millions/ml were incubated at 15 and 25°C with and without the addition of 5% seminal plasma. Duplicate motility observations on four ejaculates showed that seminal plasma completely protected the spermatozoa against the dilution effect at

both temperatures. Without added plasma, however, the effects of dilution were more marked at 25°C.

In view of the beneficial action of seminal plasma, extracts of four samples were tested. The effect of 10% (v/v) plasma, heated plasma, ethanol-extracted plasma, and dialysed plasma on fowl spermatozoa at a concentration of 0.2 million/ml is shown in Table 4. From the analysis of variance, the standard error of the difference between the treatment and control means was found to be 2.1 (d.f. = 45) and has been used for the estimation of *t*. Ethanol extracts and dialysed samples depressed motility, but heated plasma was as effective as the untreated material in preventing the effects of dilution.

TABLE 4
EFFECT OF EXTRACTS OF FOUR SEMINAL PLASMA SAMPLES ON THE MOTILITY OF DILUTED FOWL SPERMATOZOA

Mean values for four ejaculates tested on each of four samples are given

Plasma Extract	Sperm Concentration (million/ml)	Mean	Plasma Extract	Sperm Concentration (million/ml)	Mean
Nil (control)	0.2	23.9	Ethanol extract	0.2	18.1**
Whole plasma	0.2	44.8**	Dialysed plasma	0.2	16.2**
Heated plasma	0.2	44.8**	Nil (control)	20.0	54.5**

** Significantly different from control (0.2 million/ml) at $P < 0.01$.

(c) *Effect of Supernatants from Washed Spermatozoa*

To check on the possible occurrence of a protective substance in the spermatozoa which might diffuse out, supernatants from three samples of washed fowl spermatozoa were tested. The motility scores of four fowl ejaculates diluted to cell concentrations of 20 and 2 millions/ml are given in Table 5. Neither of the supernatants had any beneficial effect, in fact those prepared by freezing consistently depressed motility at the lower cell concentration.

(d) *Effects of Various Substances on Diluted Spermatozoa*

Table 6 shows the effect of a number of seminal constituents and other possible beneficial substances on the motility of fowl spermatozoa at a cell concentration of 0.2 million/ml. No analysis was considered necessary in experiment A as motility was usually zero. In experiment B, the treatments giving zero or near zero scores were omitted in the analysis of variance from which a standard error of 4.8 with 77 degrees of freedom was calculated. Bovine albumin, bovine globulin, casein, and gum acacia gave some protection against the dilution effect although none of these substances were as beneficial as seminal plasma.

IV. DISCUSSION

Fowl spermatozoa rapidly become immotile at high dilutions. The range of cell concentrations over which the phenomenon appears may vary from ejaculate to ejaculate but is of the same order as reported for other species (Kennedy 1947;

TABLE 5
MOTILITY OF FOWL SPERMATOOZA IN DILUENTS CONTAINING SPERMATOOZAL SUPERNATANTS

Three sets of supernatant samples were obtained after (a) incubation and (b) alternate freezing and thawing of washed fowl spermatozoa and tested on four ejaculates. Mean motility indices are given

Cell Concentration (millions/ml)	Preparation of Supernatant	% of Supernatant		
		0	3	9
20	Incubation	54.8	51.4	47.3
	Freezing	55.1	52.4	41.7
2	Incubation	47.1	45.5	40.8
	Freezing	50.5	37.8	23.0

Summary of the Analysis of Variance

Source of Variation	Degrees of Freedom	Variance Ratio
Effect of dilution	1	53.5**
Difference in preparation	1	11.0**
Concentration of supernatant	2	36.6**
Between supernatant samples	2	3.8**
Between ejaculates	3	9.4**
First-order interactions		
Dilution \times preparation	1	5.1*
Dilution \times supernatant concentration	2	2.1
Preparation \times supernatant concentration		
Linear difference	1	17.6**
Quadratic difference	1	0.3
Sample and ejaculate interactions	26	1.6
Residual	103	63

* $P < 0.05$. ** $P < 0.01$.

Emmens and Swyer 1948; Blackshaw 1953; White 1954) and suggests it is of general occurrence with vertebrate spermatozoa. There seems to be no apparent reason why ejaculates collected under similar conditions should vary in their reaction

to dilution. Similar variability has been found by Wales and White (unpublished data, 1961) using dilute dog spermatozoa.

To offset the detrimental effect of dilution, diluents for fowl spermatozoa should have a high tonicity similar to the semen itself. The beneficial action of potassium, especially at high dilution, confirms the opinion of White (1953*b*) that potassium loss from spermatozoa occurs during dilution. Seminal plasma is very beneficial in preventing the effects of dilution and the present work indicates that it contains a heat-stable, dialysable, protective substance, possibly a low molecular weight peptide which is precipitated by ethanol.

TABLE 6

EFFECTS OF VARIOUS SUBSTANCES ON THE MOTILITY OF FOWL SPERMATOZOA DILUTED TO A FINAL CELL CONCENTRATION OF 0.2 MILLION/ML

The mean motility indices of spermatozoa at a concentration of 100 millions/ml were 44.0 and 49.8 respectively

Experiment A (6 replications)		Experiment B (8 replications)	
Substance Added	Mean Motility Index	Substance Added	Mean Motility Index
Nil (control)	3.4	Nil (control)	0.3
Seminal plasma (10%)	31.1	Seminal plasma (10%)	37.2**
Thiamine (5 mg/100 ml)	3.2	Bovine albumin (250 mg/100 ml)	19.6**
Riboflavin (5 mg/100 ml)	0.8	Bovine γ -globulin (250 mg/100 ml)	10.6*
Niacin (5 mg/100 ml)	3.2	Casein (250 mg/100 ml)	16.4**
Inositol (5 mg/100 ml)	1.2	Egg albumin (250 mg/100 ml)	7.1
<i>p</i> -Aminobenzoic acid (5 mg/100 ml)	2.8	Starch (250 mg/100 ml)	1.3
Pantothenate (5 mg/100 ml)	4.2	Gelatin (250 mg/100 ml)	0.9
Pyridoxine (5 mg/100 ml)	1.2	Acacia gum (250 mg/100 ml)	11.4*
Folic acid (5 mg/100 ml)	1.5	Glycogen (250 mg/100 ml)	0.0
Biotin (25 μ g/100 ml)	2.2	Agar (250 mg/100 ml)	0.0
Vitamin B ₁₂ (40 μ g/100 ml)	2.7	Glycine (1 g/100 ml)	0.5
Ascorbic acid (10 mg/100 ml)	0.0	Serine (1 g/100 ml)	8.4
Cytochrome <i>c</i> (2.5 mg/100 ml)	5.0	Alanine 1 g/100 ml)	2.6
Flavine adenine dinucleotide (2.5 mg/100 ml)	1.0	Creatine (50 mg/100 ml)	4.0

* Significantly beneficial at $P < 0.05$. ** Significantly beneficial at $P < 0.01$.

Since supernatants from rabbit semen left overnight give more protection than fresh seminal plasma, beneficial substances may be lost from spermatozoa during senescence (Emmens and Swyer 1948). Blackshaw (1953), in reviewing work on rabbit, ram, and bull spermatozoa, suggested that during dilution two types of substance are lost: one diffusible and the other non-diffusible. On this basis, one might expect that supernatants prepared by incubating carefully washed spermatozoa would contain at least the diffusible substance. Supernatants prepared from sper-

matozoa damaged by freezing should be even more effective because of their content of non-diffusible substances. In the present studies, however, frozen and thawed cells apparently released a substance which further depressed the motility of diluted fowl spermatozoa.

Lorenz and Tyler (1951) found that diluted fowl spermatozoa remained motile for a longer period in the presence of 0.003–0.133M glycine. In the present studies, 0.131M glycine did not improve the motility score of highly diluted fowl spermatozoa over 4 hr. Tests with other miscellaneous substances gave results similar to those found for mammalian spermatozoa (Emmens and Swyer 1948; Blackshaw 1953; White 1954). The vitamins were inactive and proteins beneficial.

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STUDIES ON MARSUPIAL NUTRITION

III. THE COPPER-MOLYBDENUM-INORGANIC SULPHATE INTERACTION IN THE ROTTNEST QUOKKA, *SETONIX BRACHYURUS* (QUOY & GAIMARD)

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Summary

An investigation was commenced to determine whether induced copper depletion caused the seasonal anaemia known to occur in two populations of the quokka, *Setonix brachyurus* Quoy & Gaimard, on Rottnest I., W.A. Long-term feeding of molybdenum and inorganic sulphate depressed liver and blood copper levels but anaemia was not produced. In experimental quokkas a relationship was found between absolute daily copper intake and copper levels in the faeces.

Blood samples were collected from adult quokkas captured in two study areas on Rottnest I. during 1958-59. Quokkas from West End usually had higher blood copper levels and lower blood molybdenum levels than those from Lake Bagdad. Higher plant molybdenum levels at Lake Bagdad account for the higher blood molybdenum levels in these quokkas than in West End quokkas. Differences in blood copper levels between quokkas living in the two areas are not accounted for by differences in copper intake, as faecal copper levels of quokkas caught in the two areas were similar at the same times of the year. A negative correlation was found to exist between blood copper and blood molybdenum levels in Lake Bagdad quokkas, but not in those from West End. This and the experimental findings suggest that in the former blood copper levels were depressed by a higher molybdenum intake.

A positive correlation was found to exist between blood copper and haemoglobin levels in Lake Bagdad quokkas but not in West End quokkas. Although this implies that copper depletion is associated with the seasonal anaemia, mean blood copper levels of Lake Bagdad quokkas at the end of summer were similar to mean blood copper levels of the experimental quokkas, and no experimental anaemia was produced. Induced copper depletion alone does not cause the seasonal anaemia but it is one factor associated with it in Lake Bagdad quokkas and not in West End quokkas.

I. INTRODUCTION

The quokka, *Setonix brachyurus* Quoy & Gaimard, is a small herbivorous marsupial (adult weight 2-4 kg) belonging with other grazing marsupials to the family Macropodidae. Its distribution is confined to south-west Western Australia. At the time of European settlement in the early nineteenth century, the range of the quokka extended from Esperance on the south coast to the Moore R. on the west coast (Shortridge 1909). It was found on Bald I. on the south coast, 40 miles east of Albany, and on Rottnest I. on the west coast, 13 miles west of Fremantle. At

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present small populations occur in isolated pockets of the former mainland range (Barker, Main, and Sadleir 1957). Both island populations are large.

Because of the suitability of the area (Main 1959) and the lack of information on wild marsupial populations in general, a field study was commenced on the resident quokka population of Rottnest I. in November of 1953. In January of 1954 it was observed that approximately 10% of the quokkas captured in the field were emaciated. Haemoglobin estimations were carried out on field quokkas and on domesticated Rottnest I. quokkas. The haemoglobin levels of field quokkas were significantly lower than those of domesticated quokkas. Initially, field quokkas were captured only in the Lake Bagdad area (Barker, unpublished data). Later Shield (1958) and Main, Shield, and Waring (1959) showed that quokkas in two areas on Rottnest I. (Lake Bagdad and West End) undergo a seasonal anaemia which is most severe at the end of summer. In field quokkas there is a reduction in mean haemoglobin levels of 25% below the mean haemoglobin levels (16 g/100 ml) of well-fed domesticated Rottnest quokkas. There is a corresponding fall in haematocrit and red blood cell count. During the late winter and early spring, haematological levels of field quokkas recover to levels comparable with those of fed domesticated quokkas.

Since it was known that (i) sheep grazing on Rottnest in the 1920's had quickly developed "coast disease" (Rottnest Island Board of Control Files) which is now known to be a dual deficiency of copper and cobalt (Marston 1938), (ii) the quokka has ruminant-like digestive features (Moir *et al.* 1954; Moir, Somers, and Waring 1956), and (iii) anaemia and emaciation are both features of "coast disease" (Filmer 1933), the hypothesis was advanced for test that the emaciation and low haemoglobin levels observed in the Rottnest quokkas were caused by a deficiency of copper or cobalt or of both.

Copper deficiency in sheep can be caused by a low intake of copper (Bennetts and Chapman 1937; Bennetts and Beck 1942) or it can be induced as a result of feeding high levels of molybdenum and inorganic sulphate (Dick 1953*b*, 1956; Wynne and McClymont 1955, 1956). Preliminary analyses of Rottnest plants showed that some species contained very low levels of copper, moderate levels of molybdenum, and high levels of inorganic sulphate (Barker, unpublished data). In view of this finding, an experiment was carried out to test the effect of inorganic sulphate on molybdenum excretion by the quokka. It was found that, as in the sheep (Dick 1953*a*), inorganic sulphate caused a fall in blood molybdenum levels with a corresponding increase in the amount of molybdenum increased in the urine (Barker 1960*a*). Experiments to determine the long-term effect of increased molybdenum and inorganic sulphate intakes on liver and blood copper levels in the quokka were carried out. At the same time, field studies were commenced to determine whether an induced copper deficiency caused the seasonal anaemia in the Rottnest quokkas.

Experimental attempts were made to induce cobalt deficiency by feeding quokkas a low cobalt diet (Barker 1960*b*). Results from these experiments and from the field work carried out by Shield (1958) and Barker (1960*b*) suggest that cobalt deficiency is not directly associated with the seasonal anaemia in the Rottnest

quokka. The possibility will be considered herein that the seasonal anaemia is caused by an induced copper deficiency. The two previous papers in this series dealt with ruminant-like features of digestion found in the quokka (Moir, Somers, and Waring 1956) and with rate of passage and digestibility of food by the quokka (Calaby 1958).

II. METHODS

(a) *Experimental Procedure*

Twelve adult male Rottneest quokkas were housed in individual pens. They were fed a diet consisting of the following ingredients by weight: 4 parts bran; 4 parts pollard; 4 parts crushed oats; 2 parts powdered skimmed milk; $\frac{1}{4}$ part meat meal; a supplement containing 1.3 i.u. of vitamin A/g. This diet* contained 7.5 p.p.m. copper, 0.6 p.p.m. molybdenum, and 0.5 g/100 g inorganic sulphate, all on a dry weight basis. Three weeks were allowed for stabilization and then a liver biopsy was performed on each animal. After the operation the quokkas were placed in individual cages in an animal house to recuperate for 1 week before the experiment was commenced. Food was presented every day in galvanized feeders (Calaby 1958) and tap water was given to drink.

The animals were divided randomly into two groups of six. The experimental group was given a daily drench of 2 ml of a solution providing 0.5 g inorganic sulphate as ammonium sulphate and 434 μ g of molybdenum as ammonium molybdate. The drench was calculated to provide a daily intake level of 5 p.p.m. molybdenum and 1 g/100 g inorganic sulphate on the assumption that an adult male quokka eats 100 g dry weight of food daily.† The control group was given a daily drench of 2 ml of distilled water to simulate handling effects in the experimental group.

At day 0 and thereafter at 5-weekly intervals, the quokkas were weighed and 8-ml blood samples were collected by heart puncture through all-stainless steel needles and dry glass syringes. The blood was oxalated with 2 mg/ml of the ammonium-potassium oxalate mixture of Dacie (1956) and erythrocyte counts, haemoglobin estimations, haematocrit readings, and copper and molybdenum analyses were made. Mean cell volume, mean cell haemoglobin, and mean cell haemoglobin concentration were calculated to determine changes in red cell morphology in the eventuality that an anaemia was produced. Faecal samples were collected for copper analysis from three quokkas in each group for the first 2 weeks. After 10 weeks one control quokka had to be destroyed because of accidental injury. One control quokka died after 15 weeks and one control and one experimental quokka after 18 weeks. At the end of 20 weeks all quokkas were falling off in condition but anaemia had not been induced. They were slaughtered and their livers were collected for copper and molybdenum analyses.

* Three previous attempts were made to feed quokkas a low copper diet. In every case the animals starved rather than eat an unpalatable diet. In the reported experiment the diet was readily eaten but the copper level was not low.

† This approximation was based on previous observations made on the food intakes of adult male quokkas (Barker 1960b).

(b) Field Procedures

Nine collecting trips were made to Rottnest between January 1958 and May 1959. On each trip quokkas were caught from two areas, the West End and Lake Bagdad. The localities differ in that permanent fresh water is available throughout the year only at Lake Bagdad. Vegetational differences have been described by Storr, Green, and Churchill (1959). There is a large quokka population at Lake Bagdad and a small population at West End. An equal number of quokkas was caught from each area (usually 40) by hand-net over three consecutive nights except on trip 2 when the Lake Bagdad sample was collected by long-net. Both collecting techniques have been described by Dunnet (1956). After capture the quokkas were placed in individual sacks and taken to the island laboratory. They were weighed and tagged (Ealey and Dunnet 1956), the sex was noted, and the teeth examined for age determination (Shield 1958). 8-ml blood samples were collected as previously described for haemoglobin estimations and copper and molybdenum analyses. The quokkas were released at the capture site on the next day and their faeces collected from the sacks in which they had remained overnight. The results obtained from adults only are presented herein.

(c) Liver Biopsy

The quokkas were anaesthetized with ether. An incision was made in the posterior intercostal space on the right side to expose the liver. Approximately 1 g of the end of the elongate right lobe was excised with a scalpel and the cut surface sealed with a platinum cautery. Throughout the operation rigid aseptic precautions were observed. All quokkas survived the operation.

(d) Preparation of Samples for Analysis

Liver, food, and faecal samples were dried in an oven at 100°C for 18 hr and then ground in an all-steel mill. Blood samples were measured into 100-ml Kjeldahl flasks and a few millilitres of glass-distilled water added. The flasks were heated over a gas flame to partially dry the samples. Drying was completed by heating overnight in an oven at 100°C. All samples were wet digested with glass-distilled sulphuric, nitric, and perchloric acids.

(e) Chemical Methods

Copper was estimated by the method of Eden and Green (1940) and molybdenum by the method of Piper and Beckworth (1948). Inorganic sulphate was estimated by the method of Dick and Bingley (Dick 1954), the end-point being determined by titration.

(f) Haematological Procedures

Red cell counts were recorded by a photographic technique adapted from the methods of Brown (1953) and Swisher and Izzo (1953). The blood sample was diluted in the formalin-trisodium citrate diluting fluid of Dacie (1956). Haemoglobin estimations were carried out by the oxyhaemoglobin method (Dacie 1956). Haematocrit readings were made using Baird and Tatlock microhaematocrit tubes spun in metal sheaths for 30 min at 3000 r.p.m. No correction was made for trapped plasma.

TABLE 1
PROGRESSIVE MEAN HAEMATOLOGICAL LEVELS OF THE TWO GROUPS OF QUOKKAS USED IN THE REPORTED EXPERIMENT

Group	Day	Haemoglobin (g/100 ml)	Haematocrit (%)	Red Blood Cells ($\times 10^6/\text{mm}^3$)	Mean Cell Volume (μ^3)	Mean Cell Haemoglobin ($\mu\mu\text{g}$)	Mean Cell Haemoglobin Concn. (g/100 ml)	Copper ($\mu\text{g}/\text{ml}$)	Molybdenum ($\mu\text{g}/\text{ml}$)
Control (6)* Experimental (6)	0	14.3	37.6	6.00	62.9	24.0	38.1	0.63	0.03
	0	14.1	37.9	6.01	63.0	23.6	37.3	0.68	0.05
Control (6) Experimental (6)	35	15.8	42.4	7.29	58.2	21.7	37.2	0.52	0.06
	35	15.9	43.4	7.45	58.4	21.4	36.4	0.25†	0.15‡
Control (6) Experimental (6)	70	16.2	43.3	7.57	57.3	21.5	37.5	0.52	0.05
	70	15.6	42.4	7.26	58.6	21.6	36.7	0.18	0.12
Control (4) Experimental (6)	105	16.7	44.3	7.93	56.1	21.1	37.6	0.42	0.05
	105	14.9	41.1	7.02	58.6	21.2	36.2	0.18	0.13
Control (3) Experimental (5)	140	15.1	42.0	7.32	57.6	20.7	36.0	0.43	0.07
	140	14.8	40.9	7.05	58.1	21.0	36.1	0.20	0.16

* The numbers of animals remaining alive in the groups are given in parenthesis.

† Analysis of variance gave an error variance on 10 d.f. = 0.0444 ($\mu\text{g}/\text{ml}$)². Variance ratio between groups on 1 and 10 d.f. = 50.49 ($P < 0.001$).

‡ Analysis of variance gave an error variance on 10 d.f. = 0.00012 ($\mu\text{g}/\text{ml}$)². Variance ratio between groups on 1 and 10 d.f. = 195.0 ($P < 0.001$).

III. RESULTS

(a) *Experimental*

(i) *Effect of Increased Molybdenum and Inorganic Sulphate Intake on Blood and Liver Copper Levels.*—The mean results of the blood analyses carried out on the control and experimental groups are presented in Table 1, and those of the liver analyses are shown in Table 2. After 35 days, blood copper levels of the experimental group had fallen significantly below those of the control group. The trend was maintained during the remainder of the experiment. Similarly, the liver copper levels of the experimental group were significantly lower than those of the control group at the end of the experiment.

TABLE 2
MEAN PRE- AND POSTEXPERIMENTAL LIVER COPPER AND MOLYBDENUM
LEVELS OF THE TWO GROUPS OF QUOKKAS USED IN THE REPORTED EXPERIMENT
Results expressed in p.p.m. on a dry weight basis

Group	Liver Copper Level		Liver Molybdenum Level	
	Before Experiment	After Experiment	Before Experiment	After Experiment
Control	14.0	14.7	7.5	6.0
Experimental	12.9*	8.6*	6.7	6.7

* Analysis of variance gave an error variance on 10 d.f. = 0.527 (p.p.m.)². Variance ratio between groups on 1 and 10 d.f. = 212.97 ($P < 0.001$).

At the beginning of the experiment, blood molybdenum levels were higher in the experimental group than in the control group of quokkas. However, during the experiment blood molybdenum levels in the experimental group of quokkas rose markedly, whereas there were only slight increases in the control group. There was no statistical difference between the liver molybdenum levels of the two groups at the end of the experiment.

In spite of the pronounced fall in blood copper levels in the experimental group, the red cell counts, haemoglobin levels, and haematocrit readings did not fall and there were no significant differences between the groups in these respects. The increases in these values in both groups during the first 35 days were probably due to recovery from the liver biopsy. During the operation all quokkas had haemorrhaged from the cut surface of the liver. There was no difference between the groups in erythrocyte morphology as shown by mean cell volume, mean cell haemoglobin, or mean cell haemoglobin concentration.

(ii) *Relationship between Copper Intake and Copper Levels in the Faeces.*—Daily faecal collections were made from three quokkas in each group of the described

experiment, for the first 2 weeks, and from six quokkas fed a high copper diet in a previous experiment (Barker 1960*b*). Food and faecal samples were analysed for copper. From the results, calculated 24-hr copper intakes were statistically equated to faecal copper levels. A correction of 2 days was made to allow for rate of passage of food, i.e. the absolute copper intake of day 1 was equated to the faecal copper level of day 3. Calaby (1958) found a rate of passage of approximately 2 days for a similar type of diet fed to quokkas. The correction applied in the present case is the best approximation available. From the results of 165 observations the following regression was calculated:

$$y = 0.0168x + 0.344,$$

where y = daily copper intake in mg, and x = 24-hr faecal copper levels in p.p.m. on a dry weight basis. The correlation coefficient was significant ($r = 0.5$ on 163 d.f.,

TABLE 3
DETAILS OF THE SEX AND NUMBERS OF ADULT QUOKKAS CAPTURED IN TWO LOCALITIES ON
ROTTNEST I. BETWEEN 1958 AND 1959

Trip	Date	Lake Bagdad		West End		Total
		Male	Female	Male	Female	
1	17. i.58	8	3	2	6	19
2	22. ii.58	4	5	10	11	30
3	11. iv.58	12	14	14	12	52
4	21. v.58	7	13	8	12	40
5	2.viii.58	8	8	10	8	34
6	15. x.58	7	10	8	7	32
7	2. xii.58	3	7	9	4	23
8	19. ii.59	2	7	5	6	20
9	30. v.59	5	7	8	9	29
Total		56	74	74	75	279

$P < 0.001$). For an example, from the equation it can be calculated that a quokka with a faecal copper level of 39 p.p.m. on a dry weight basis had an absolute daily copper intake of 1 mg. The relationship was calculated to facilitate the interpretation of field results. A similar type of relationship has been calculated in the sheep (Dick 1954).

(b) Field Work

Details of the sex and numbers of adult quokkas caught on nine collecting trips to Rottneest between January 1958 and May 1959 are presented in Table 3. The overall sex ratio at West End was 1 : 1 while at Lake Bagdad females outnumbered males approximately 3 : 2.

(i) *Blood Copper Levels*.—The mean blood copper levels of quokkas captured in both areas on all trips are presented in Figure 1. Analyses of variance showed

that blood copper levels of Lake Bagdad quokkas were significantly lower than those of West End quokkas on all but three trips.

(ii) *Blood Molybdenum Levels.*—The mean blood molybdenum levels of quokkas captured in both areas on all trips are presented in Figure 2. Analyses of variance

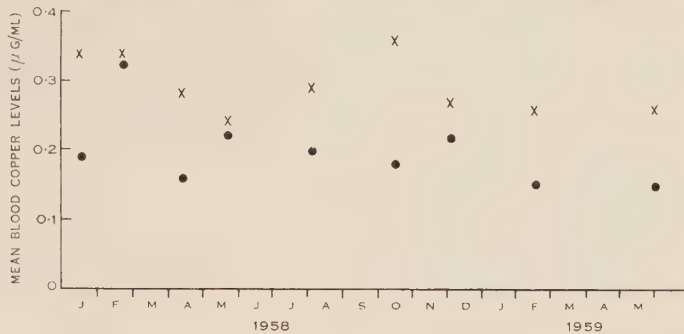


Fig. 1.—Mean blood copper levels of quokkas captured at Lake Bagdad (●) and West End (×) during 1958 and 1959 expressed in $\mu\text{g/ml}$ whole blood. Analyses of variance showed that blood copper levels of Lake Bagdad quokkas were significantly lower than those of West End quokkas in January 1958 ($P < 0.001$), April 1958 ($P < 0.01$), August 1958 ($P < 0.05$), October 1958 ($P < 0.001$), February 1959 ($P < 0.01$), May 1959 ($P < 0.05$). On the remaining three trips there was no significant difference between blood copper levels.

showed that blood molybdenum levels of Lake Bagdad quokkas were significantly higher than those of West End quokkas on all but two trips.

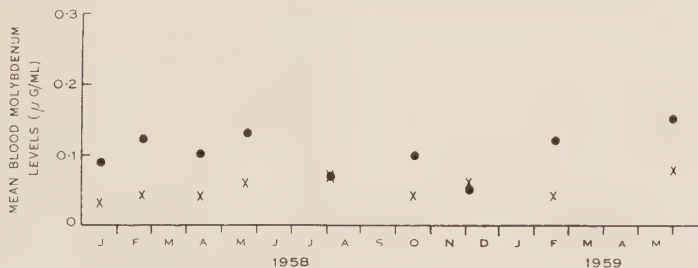


Fig. 2.—Mean blood molybdenum levels of quokkas captured at Lake Bagdad (●) and West End (×) during 1958 and 1959 expressed in $\mu\text{g/ml}$ whole blood. Analyses of variance showed that blood molybdenum levels of Lake Bagdad quokkas were significantly higher than those of West End quokkas in January 1958 ($P < 0.001$), February 1958 ($P < 0.001$), April 1958 ($P < 0.001$), May 1958 ($P < 0.01$), October 1958 ($P < 0.001$), February 1959 ($P < 0.01$), May 1959 ($P < 0.001$). On the remaining two trips there was no significant difference between blood molybdenum levels.

(iii) *Haemoglobin Levels.*—The mean haemoglobin levels of quokkas captured in both areas on all trips are presented in Figure 3. West End quokkas had significantly lower haemoglobin levels than Lake Bagdad quokkas at the end of summer

(April) in 1958. At the end of winter and in the spring of 1958, mean haemoglobin levels of West End quokkas were higher than those of Lake Bagdad quokkas. The levels were significantly higher only in August. This pattern of haemoglobin levels throughout 1958, in both areas, is similar to that found by Shield (1958) in the previous three years. That is, haemoglobin levels of West End quokkas were lower at the end of summer and higher in the spring than haemoglobin levels of Lake Bagdad quokkas. However, at the end of summer 1959 the pattern was reversed and West End quokkas had significantly higher levels than those of Lake Bagdad quokkas.

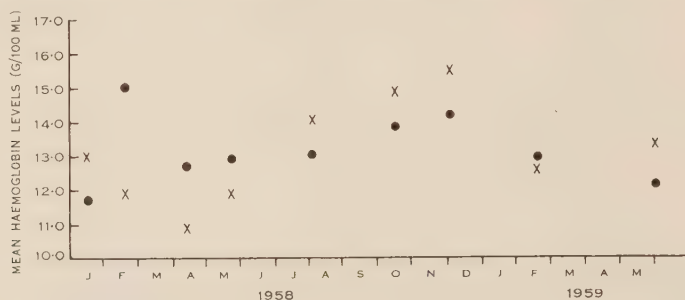


Fig. 3.—Mean haemoglobin levels of quokkas captured at Lake Bagdad (●) and West End (×) during 1958 and 1959 expressed in g/100 ml. Analyses of variance showed that haemoglobin levels of West End quokkas were significantly lower than those of Lake Bagdad quokkas in February 1958 ($P < 0.001$), April 1958 ($P < 0.001$), and May 1958 ($P < 0.01$); haemoglobin levels of West End quokkas were significantly higher than those of Lake Bagdad quokkas in August 1958 ($P < 0.05$), and May 1959 ($P < 0.05$). On the remaining four trips there was no significant difference between haemoglobin levels.

Haemoglobin levels and blood copper levels of the Lake Bagdad quokkas, caught by long-net on trip 2, were significantly higher than those of Lake Bagdad quokkas caught by hand-net on trips 1 and 3 (see Figs. 1 and 3). It has yet to be determined which capture technique gives the most representative sample of quokkas from the population.

(iv) *Relationship between Haemoglobin and Blood Copper Levels.*—Statistical analysis showed that a positive relationship existed between haemoglobin and blood copper levels in Lake Bagdad quokkas but not in West End quokkas. The following regression was obtained for Lake Bagdad quokkas:

$$y = 4.06x + 12.24,$$

where y = haemoglobin level in g/100 ml, and x = whole blood copper level in $\mu\text{g/ml}$. (Error variance on 124 d.f. = 2.152. Variance ratio on 1 and 124 d.f. = 8.45 ($P < 0.001$).)

(v) *Relationship between Blood Molybdenum and Blood Copper Levels.*—A negative relationship was found to exist between blood molybdenum and blood copper levels in Lake Bagdad quokkas but not in West End quokkas. The following regression was obtained for Lake Bagdad quokkas:

$$y = -0.26x + 0.22,$$

where y = whole blood copper level in $\mu\text{g/ml}$, and x = whole blood molybdenum level in $\mu\text{g/ml}$. (Error variance on 124 d.f. = 0.0086. Variance ratio on 1 and 124 d.f. = 4.299 ($P < 0.05$).)

(viii) *Comparison between Faecal Copper Levels of Quokkas Captured in Two Areas at the same Times of the Year.*—It has been shown that under experimental conditions a relationship existed between daily copper intake and faecal copper levels in the quokka. Faecal copper levels of quokkas caught in the two study areas at the same times of the year were compared to assess the differences in copper intakes. Eighty-seven faecal samples from adult quokkas caught in May and October 1958 and May 1959 were analysed. These samples were collected at times of the year when the quokkas were in their best physical condition (October) and worst (May) in both areas. The results of statistical analyses carried out on the data are presented in Table 4. There were no significant differences between the faecal copper levels of the quokkas collected in the two areas on these three occasions.

TABLE 4

COMPARISON BETWEEN MEAN FAECAL COPPER LEVELS OF QUOKKAS CAPTURED ON TRIPS 4, 6, AND 9
Results expressed in p.p.m. on a dry weight basis

Trip	Date	Lake Bagdad	West End	D.F.	t	P
4	21.v.58	11.04	11.81	31	0.40	N.S.
6	15.x.58	9.20	10.36	29	1.05	N.S.
9	30.v.59	12.72	11.17	21	0.88	N.S.

IV. DISCUSSION

The results from the experiment described herein clearly show that molybdenum and inorganic sulphate, at the levels fed, exert a depressing effect on blood and liver copper levels in the quokka. In this respect the quokka resembles the sheep (Dick 1953*b*, 1953*c*; Wynne and McClymont 1955, 1956) and cow (Mylrea 1958). Under the conditions of the experiment copper deficiency anaemia was not induced. Work relating to the interaction between copper-molybdenum-inorganic sulphate in both ruminants and non-ruminants has been reviewed by Miller and Engel (1960). Dick (1956) advanced the hypothesis "that a membrane whose permeability to molybdenum is impeded or blocked by sulphate impedes or blocks copper transport" (op. cit. p. 471). Scaife (1956) failed to show any effect by inorganic sulphate on the rate of diffusion of molybdenum across the red cell membrane. Mills (1960) studied the effect of molybdenum and inorganic sulphate on rumen and abomasal levels of sulphide and soluble copper, and on the excretion of ^{64}Cu in the sheep. His results suggest that the interaction affects the absorption rather than the excretion of copper. The preliminary nature of the reported investigation precluded any work being carried out on the mechanisms involved in the copper-molybdenum-inorganic sulphate interaction.

On all trips to the island when quokkas were captured for blood sampling, plant samples were collected from the two study areas for analysis of copper, molybdenum, and inorganic sulphate levels. The results of plant analyses have been presented elsewhere (Barker 1961). Briefly the findings were that plant copper levels were very low in both areas. The majority of samples had levels of less than 3.0 p.p.m. copper on a dry weight basis. In sheep pastures such levels are classified as deficient by Bennetts and Beck (1942). In both study areas some plants had inorganic sulphate levels of up to 2.0 g/100 g. The normal inorganic sulphate levels of Western Australian mainland pastures used for grazing sheep range from 0.2–0.3 g/100 g (Beck, personal communication). Plant molybdenum levels were of the order of 1.0 p.p.m. at West End and 5.0 p.p.m. at Lake Bagdad, both on a dry weight basis. There are no figures available for molybdenum levels in Western Australian mainland plants. A summary of all the field findings is presented in Table 5.

TABLE 5
SUMMARY OF THE FINDINGS AT LAKE BAGDAD AND AT WEST END DURING 1958 AND 1959

	West End	Lake Bagdad
1. Fresh water	None	Permanent
2. Population density	Low	High
3. Plant copper levels	Low	Low
4. Plant molybdenum levels	Low	Moderate
5. Plant inorganic sulphate levels	High	High
6. Faecal copper levels	Low	Low
7. Blood copper levels	Moderate	Low
8. Blood molybdenum levels	Low	Moderate
9. Haemoglobin levels at the end of summer	Low	Low
10. Correlation between blood copper and blood molybdenum levels	Not significant	Significant, negative
11. Correlation between haemoglobin and blood copper levels	Not significant	Significant, positive

The differences in blood molybdenum levels between quokkas captured at West End and at Lake Bagdad can be attributed to differences in molybdenum levels of the plants eaten in the two areas. However, the observed differences in blood copper levels between quokkas captured in the two areas cannot be attributed to differences in the copper levels of the food plants. The data from faecal copper analyses of field quokkas indicate that copper intakes of quokkas in both areas were similar on the three occasions when faecal copper levels were compared. It can be inferred from the negative regression calculated between blood copper levels and blood molybdenum levels in Lake Bagdad quokkas, that increased blood molybdenum levels were directly associated with decreased blood copper levels. This evidence, taken in consideration with the experimental finding that increased molybdenum and inorganic sulphate intakes depress blood copper levels, suggests that blood copper levels in Lake Bagdad quokkas were lower than those in West End quokkas because in the former animals molybdenum intake was higher.

The positive regression calculated between blood copper levels and haemoglobin levels in Lake Bagdad quokkas shows that lowered blood copper levels were associated with low haemoglobin levels. This finding indicates that an induced copper depletion is linked with the seasonal anaemia in Lake Bagdad quokkas, but not in West End quokkas. However, there is an apparent discrepancy between the field and experimental findings. The mean blood copper levels of the experimental group of quokkas used in the reported experiment at week 5 and onwards were comparable to mean blood copper levels of Lake Bagdad quokkas at the end of summer, yet an experimental copper deficiency anaemia was not produced. Further examination of the data shows that there was a larger variation in blood copper levels of Lake Bagdad adult male quokkas at the end of summer (range 0.03–0.33 $\mu\text{g/ml}$ on trips 3 and 9) than in the experimental group (range 0.14–0.27 $\mu\text{g/ml}$ from week 5 onwards). As the level of blood copper below which a quokka can be considered copper deficient is not known, no statement can be made on the percentage of Lake Bagdad quokkas which can be considered copper deficient at the end of summer.

Under the experimental conditions used, the intake of copper, and possibly protein, was higher than in field quokkas. The comparison between copper intakes can be made on the basis of faecal copper levels. In experimental quokkas the mean faecal copper level was 25 p.p.m. while Lake Bagdad quokkas had mean faecal copper levels of 11.7 p.p.m. in May 1958 and 12.7 p.p.m. in May 1959. No direct comparison can be made between the protein intakes of field and experimental quokkas. The experimental diet was not analysed for protein and as yet no direct assessment has been made of the protein intakes of field quokkas. It seems reasonable to assume that protein intake of quokkas fed grain concentrates and on which they gained weight, would be higher than that of field quokkas eating plants considered inadequate in protein during the summer (Main, Shield, and Waring 1959) and on which they lose weight (Shield 1958; Barker 1960*b*). Further work is required to determine how the copper–molybdenum–inorganic sulphate interaction in the quokka is affected by varying protein intakes.

Although it cannot be stated that copper deficiency *causes* the seasonal lowering of haematological levels in Lake Bagdad quokkas, the results strongly suggest that a lowered copper status, induced by the copper–molybdenum–inorganic sulphate interaction, is one factor *associated* with the annual haematological decline in Lake Bagdad quokkas but not in West End quokkas.

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MITOTIC ACTIVITY IN WOOL FOLLICLE BULBS

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Summary

The quantitative significance of mitosis in wool follicle bulbs in relation to the rate of fibre growth was investigated in 10 major fleece phenotypes (fine Merino, medium Merino mosaic, strong Merino, Merino lustre mutant, Corriedale carpet-wool, Corriedale mosaic, Southdown, and Border Leicester). Mitotic activity in the follicle bulbs was estimated with the aid of colchicine and the volume rate of fibre growth was inferred from fibre diameter.

From the quantitative relationships between fibre diameter and the number of mitoses occurring in a bulb, it was concluded that at least two-thirds of the differences in the volume rate of fibre growth were due to difference in the number of cortical cells produced in the bulb. The remainder of the differences in fibre growth were referable to variations in cortical cell volume. Compared with small fibres therefore, large fibres contained more cortical cells and these cells were also larger. The correlation coefficient of fibre diameter with mitoses¹ between fibres within phenotypes was $r = +0.67$ and between means of phenotypes was $r = +0.95$ (after 3 hr colchicine).

The correlation coefficients of mitoses¹ with bulb diameter between follicles within phenotypes was $r = +0.71$ and between means of phenotypes was $r = +0.97$ (after 3 hr colchicine). In the absence of evidence that the germinal cells in the bulb differed greatly in size it was concluded that differences in the number of cortical cells produced were due to differences in the number of germinal cells rather than to differences in cell turnover rate, although some evidence was obtained for small differences in turnover rate.

The capacity of the skin follicle as an organ of protein synthesis is discussed. Within the follicle, most of the new material is probably incorporated in the proliferative phase of fibre growth rather than in the keratinization phase. It is concluded that the keratinization phase is probably not a rate-limiting process of fibre growth, but that the proliferation phase is rate limiting.

1. INTRODUCTION

Mammalian hair and wool fibres are composed of cells derived from the follicle bulb and subsequently subjected to the physicochemical changes associated with keratinization. The mitotic activity giving rise to the cellular basis of the various fibre components (medulla, cortex, and cuticle) is restricted to the follicle bulb, i.e. the cellular matrix which surrounds the dermal papilla. This fact is apparent in the illustrations of Segall (1918) and has been emphasized by Pinkus (1927), Auber (1952), Chase (1954), and Bullough and Laurence (1958). However, there appear to have been few quantitative studies of mitotic activity in the follicle bulb—particularly as it relates to the rate of fibre growth. Hoffman (1953) gives the cell turnover time during the growth period in the human hair bulb as 3 days. Kiljunen (1956) observed

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that cyclic phases of hair growth in rats were closely associated with changes in mitotic activity in the follicle bulb. Bullough and Laurence (1958) have extensively investigated factors influencing mitotic activity in the bulb in mice skin under both *in vivo* and *in vitro* conditions. Hopsu and Harkonen (1960) observed that mitotic activity in the bulb in the mouse rose to a maximum on the 6th–8th day of the hair growth cycle, which was initiated by plucking.

The object of the present investigation was to determine quantitatively the extent to which cell multiplication contributes to fibre growth in sheep. For this purpose sheep producing widely different types of fleece were chosen. Close relationships were found between the diameter of a fibre and the number of mitoses in the follicle bulb which produced it, and between the number of mitoses and the size of the follicle bulb. These relationships were sufficiently close to accept mitotic activity in the bulb as a test system for studying the mechanisms controlling fibre growth.

II. EXPERIMENTAL

(a) General

Fibre growth is growth of the exfoliative kind, i.e. growth in which the cells are removed, and enter processes of differentiation, at the same rate as they are produced. The number of cells in the germinal population located in the follicle bulb remains unchanged in number. This must be so, on the average, in a population of cells in a steady state and in which division is asynchronous, although it may not be absolutely correct over very short periods of time.

A priori, the rate of fibre growth is determined by the number of cells produced per unit time, by the size of these cells, and by the rate of production of intercellular substance. Because the total amount of intercellular substance is relatively small it is unlikely to be an important source of difference in fibre size and hence will not be considered further here. Also, in view of the difficulties of measurement of cell size (Hoffman 1953) no systematic attempt was made to measure this character directly.

The number of cells produced in a given time is a function of the number of cells in the parent population and the rate at which cells divide.

Determination of the number of cells in a follicle bulb is difficult. In these observations it was inferred from the diameter of the follicle bulbs: a measure which has already been shown to be related to fibre growth (Rudall 1956). We have no information on the accuracy of bulb diameter as an index of the number of parent cells; any inaccuracy will be a consequence of differences in mean parent cell size in different bulbs and in the shape of the bulbs.

The mitotic index (M.I.) is commonly used as the standard measure of mitotic activity and is defined as the ratio of the number of cells in mitosis (M) to the number of cells in the population being examined (N), i.e. M/N ; it is determined by the ratio of the duration of mitosis (m) to the intermitotic period (t). Obviously any factor that causes a change in the time relationships of the mitotic cycle will cause a change in M.I. unless m and t are affected to the same degree. M.I. is not necessarily a satisfactory measure of mitotic rate in the absence of other information on the time

relationships of the cycle, as emphasized by Evans, Neary, and Tonkinson (1957). Determinations of these time relationships are exceedingly difficult, and usually cannot be made by direct observations *in vivo* (Hoffman 1953).

Estimation of the number of cells produced in a given time is most readily carried out by the colchicine method. Colchicine arrests mitosis at the metaphase stage (Dustin 1934; Ludford 1936; Hawkes 1944). With adequate dose levels it neither stimulates nor inhibits the entry of cells into mitosis (Brues and Cohen 1936; Ludford 1936; Barber and Callan 1943; Bullough 1949; Brues 1951). Using mouse ear epidermis Bullough (1949) concluded that the entry of cells into mitosis was inhibited by 0.1 mg colchicine in adult mice after about 5 hr. With higher dose rates, there was an inhibition of entry of cells into mitosis (Bullough 1949; Brues 1951) while with low dose rates, this inhibition was delayed (Bullough 1949). Because of the toxic side effects of colchicine it was necessary first to determine the appropriate dose levels for sheep. The relevant experiments are described below.

Fibre growth was inferred from fibre diameter. This assumes a high positive correlation between fibre diameter and fibre volume. This is reasonable because of the high correlation between length and diameter, both between fleece types (Darlow and Craft 1935; Galpin 1948; Daly and Carter 1955; Oczan 1956) and between fibres of the same region (Galpin 1948; Oczan 1956).

(b) *Methods*

(i) *Histological Procedures*.—Biopsy skin specimens were fixed in Zenker-acetic for 4–6 hr, washed in running water for 12–14 hr, placed in iodized 50% and 70% alcohol for 12–24 hr and stored in 70% alcohol while awaiting processing. Specimens were embedded in paraffin and 40–80 serial sections prepared at 8 or 10 μ . These were cut right angles to the skin surface and, as far as possible, in the plane of the long axis of the majority of follicles. Sections were stained with haematoxylin, eosin, and picric acid or with crystal violet and eosin. For routine preparation of sections for counting mitotic figures, the crystal violet staining was particularly useful, because nuclei in mitosis are so clearly stained. After some early difficulties the following technique was found to be very satisfactory: deparaffinize and bring down to water; treat in 1N HCl at 37°C for 10 min; rinse in water; stain 1% aqueous crystal violet 1 min; rinse in water; Lugol's iodine 1 min; rinse in water; decolorize in 70% alcohol until no more violet stain runs off section; 5–6 dips in acid alcohol; wash in water; counterstain in eosin; dehydrate, clear, and mount.

(ii) *Mitotic Counts*.—All counts were made with the aid of a microscope rather than with a microprojector. For some purposes it is necessary to estimate the proportion of split nuclei in a count. This was done by reconstruction of serial sections from camera lucida drawings, using a Zeiss-Winkel drawing apparatus. The formulae of Abercrombie (1946) and Storey and Leblond (1951) were not considered applicable to the present situation because these formulae assume spherical nuclei, whereas colchicine-blocked metaphases, which form by far the greater proportion of mitotic figures counted, are discoid. The results of the observations on split nuclei are given in Table 1. The proportion of nuclei split at sectioning was about 16% but there was

substantial variation between individual follicles. If sections were cut at $10\ \mu$ rather than $8\ \mu$, this proportion would probably fall to 12–13%. For routine purposes it is not essential to correct for split nuclei as it is unlikely to be a major source of error unless comparisons are made between different tissues and absolute counts are required.

TABLE 1

OBSERVATIONS ON THE PROPORTION OF NUCLEI IN MITOSIS "SPLIT" IN TWO OR MORE SECTIONS
Sections cut at $8\ \mu$, sheep E302

Series*	Fibre Diameter† (μ)	No. of Mitoses‡	"Split" Mitoses		No. of Histological Sections
			No.	%	
1	18	22	5	23	9
	20	14	2	14	11
	23	13	3	23	9
	30	52	11	21	15
	34	40	1	2.5	17
	35	30	6	20	17
Mean	26.5	28.5	4.7	16.5	13
2	14	11	1	9	9
	15	8	2	25	8
	16	21	1	5	8
	18	16	1	6	11
	18	28	4	14	9
	19	23	4	17	10
	20	31	7	22	12
	22	18	2	11	9
	23	44	4	9	11
	24	24	6	25	12
	26	27	1	4	8
	29	81	12	15	18
	34	94	20	21	15
	37	79	10	12	16
Mean	22.5	36	5.4	14.9	11.1

* Series 1 observations were made on skin taken prior to administration of colchicine, series 2 observations 2 hr after colchicine.

† Diameter of fibre produced by bulb examined.

‡ Corrected for "split" mitoses.

Mitotic numbers were estimated in two ways: (1) total bulb counts, and (2) random bulb section counts. *Total bulb counts* were made by counting all mitotic nuclei in the serial sections through a follicle bulb. In general, counts on 20–30 follicle bulbs were satisfactory for characterizing a specimen. Selection of follicles is necessary in some animals in which bulbs may be strongly deflected and follicles

curved in more than one plane. *Random bulb section counts* were made by recording the frequency of follicles having 0, 1, 2, 3, . . . , n mitotic figures per bulb section. Every bulb was examined in a series of sections, chosen such that no single follicle was represented by more than one count. This was usually obtained by examining all the bulbs in every 10th–15th serial section (each 4–8 mm of epidermis in length) until 300–500 bulbs were counted.

(iii) *Colchicine Dose Rate Observations*.—Intravenous injections of colchicine B.P. were given to four medium-wool Merino sheep at 0920 hr and biopsy skin specimens taken at 0, 1, 2, 4, 6, and 8 hr after injection. These sheep had previously been

TABLE 2
SHEEP IDENTIFICATION, PHENOTYPE, AND COLCHICINE DOSE

Sheep No.	Phenotype	Body Weight (kg)	Colchicine (mg)	Sample Site
E127	Fine-wool Merino	38.1	7.6	Right mid-side
B451	Merino mosaic*	61.8	12.4	Rump
A288	Lustre mutant*	65.8	13.2	Right mid-side
B498	Carpet-wool Corriedale	57.5	11.5	Right mid-side
B434	Corriedale mosaic	76.7	15.5	Left shoulder
A961	Border Leicester	71.0	14.2	Right mid-side
SA4	Southdown	60.5	12.1	Right mid-side
B654	Strong-wool Merino	57.5	11.5	Left mid-side

* Mosaic phenotypes have been described by Fraser and Short (1958) and lustre mutants by Short (1958).

on a relatively low level of nutrition. The dose rates of colchicine were: sheep E302, 5 mg; E347, 10 mg; E312, 20 mg; E356, 40 mg—equivalent to 0.17, 0.33, 0.66, and 1.36 mg per kg body weight respectively.

(iv) *Investigation of Mitosis in Different Fleece Phenotypes*.—Representative animals of the major fleece phenotypes were given intravenous colchicine (B.P.) at the rate of 0.2 mg per kg and biopsy skin samples (numbers 1, 2, and 3) taken at 0, 2, and 3 hr. To reduce possible differences in mitotic rate due to differences in feed intake, sheep were individually fed a diet comprising lucerne chaff 50, crushed maize 40, coconut meal 10, NaCl 1, at the rate of 18 g per kg body weight for 8 weeks before the trial. The details of the phenotypes, the dose rates of colchicine, and the sample sites are given in Table 2. In those animals exhibiting fleece mosaicism, biopsy skin samples were taken from both normal and mosaic areas. The colchicine injections were given between 1155 and 1245 hr.

(v) *Statistical Aspects*.—In examining the relationship between mitotic number and both bulb and fibre diameter, the cube root of the mitotic number was taken to reduce this from a volume to a linear dimension. Preliminary examination showed that the transformed variate was linearly related in a statistical sense to the two diameters.

III. RESULTS

(a) *Colchicine Dose Rates*

The results of the dose rate investigations are illustrated in Figure 1. In the three sheep which received either the 10, 20, or 50 mg dose of colchicine there was a linear increase in the mean number of mitoses per random bulb section from zero time until 6 hr after injection. In the sheep which received 5 mg colchicine, no increase in the number of mitoses was detected at 1 hr: thereafter there was a linear increase until 6 hr after injection. It is possible that part of the injection in the

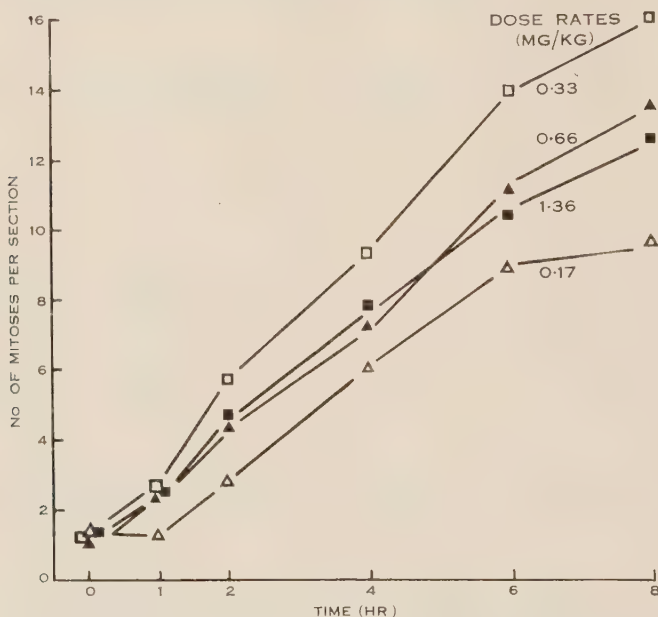


Fig. 1.—Mean number of mitoses per follicle bulb section ($8\ \mu$ thick) after different dose rates of colchicine (as indicated on figure).

sheep receiving 5 mg was lodged subcutaneously because of movement during injection. At 8 hr, the rate of accumulation of new mitoses had commenced to decline. These results agreed well with those of others which have indicated that with low dose rates of colchicine there is a delay in the arresting effect (Bullough 1949) and that after some 5–6 hr, irrespective of dose rate, there is a decline in the rate of accumulation of arrested mitoses. Bullough (1949) suggested that there was a toxic inhibition of the entry of cells into mitosis after 5 hr, but his evidence does not appear to exclude other explanations for the decline in rate of accumulation of mitotic figures after 5–6 hr. Two possible explanations are, firstly, a drop in the tissue levels of colchicine as a consequence of metabolic degradation and excretion and, secondly, actual “escape” of blocked mitoses from the metaphase arrest. Evidence against the first of these explanations is the relatively slow breakdown of colchicine (Brues 1942) and in favour of the second is the occurrence of telophase and anaphase figures in samples taken 10 hr after colchicine (Ludford 1936.) In the present observations,

telophase and anaphase figures were observed in samples taken 8 hr after administration of colchicine.

E302 (5 mg colchicine) was the only sheep to survive the colchicine trial without apparent ill effects. E347 (10 mg) showed mild diarrhoea at 3.3 hr but this did not progress: the sheep died 6 days later. E312 (20 mg) and E356 (40 mg) exhibited diarrhoea from 2.3 hr and substantial increases in respiratory rate from 4 hr: both sheep died 10–18 hr after injection of colchicine.

These observations indicated that the dose margin between full metaphase arrest and death may be narrow; future observations were made using a dose rate of 0.20 mg/kg body weight.

TABLE 3
MEANS, STANDARD DEVIATIONS, AND STANDARD ERRORS OF BULB DIAMETER AND FIBRE DIAMETER
Combined data from samples 1, 2, and 3

Sheep No.	Bulb Diameter (μ)			Fibre Diameter (μ)		
	\bar{x}	σ	S.E.	\bar{x}	σ	S.E.
B451(N)*	91.9	12.65	2.11	28.0	4.79	0.80
B451(M)†	109.3	19.62	2.92	34.3	7.30	1.09
A288	68.2	7.76	0.92	18.0	2.75	0.33
B498	120.7	28.87	3.50	38.2	8.76	1.06
B434(N)*	108.5	15.40	1.80	34.7	6.53	0.76
B434(M)†	108.2	17.95	2.65	35.1	6.90	1.02
A961	126.2	15.52	1.88	40.1	8.13	0.99
SA4	99.4	12.61	1.55	28.6	4.82	0.59
B654	92.3	14.64	1.69	25.3	6.86	0.79
E127	81.2	9.23	1.12	24.3	3.72	0.45

* N = normal area.

† M = mosaic area.

(b) Mitosis in Different Fleece Phenotypes

(i) *Variability of the Characters Studied.*—The following data from sample 2 indicate the ranges observed in the observations on 626 follicles and 10 phenotypes. The diameter of fibres ranged from 12 to 75 μ , a factor of 6.25: the means of phenotypes ranged from 17.5 to 39.4 μ , a factor of 2.25. No medullated fibres were observed in the current observations. The diameter of individual follicle bulbs ranged from 56 to 212 μ , a factor of 3.8, while the means of phenotypes ranged from 68 to 118 μ , a factor of 1.74. The number of mitoses per follicle bulb varied from 21 to 521, a factor of 24.8, while phenotypes means ranged from 32.4 to 166, a factor of 5.12.

Most of the analyses reported here are concerned with the relationships existing between these characters.

(ii) *Diameters of Follicle Bulbs and Fibres.*—There were large differences between mean bulb and mean fibre diameters (Table 3) but these two characters were closely associated with one another. The correlation coefficients between bulb diameter

and fibre diameter were: (1) between phenotypes, $r = +0.99$; (2) between follicles within phenotypes, $r = +0.57$; (3) from all the 626 follicles examined, $r = +0.80$. This latter figure is the same as that of Rudall (1956) for the correlation between fibre cross-sectional area and bulb diameter in 1500 follicles from Romney Marsh lambs 2 weeks to 6 months old.

Despite the very high correlation ($r = +0.99$) between phenotype means, phenotypes still differed in the diameter of fibre produced after adjustment for differences in bulb diameter (Table 4). However, the variance due to these deviations from regression was small, as judged from the relatively small sum of squares associated with it (Table 4, item 3), and from the high correlation between the variables (Fig. 2). The regression of means ($b = +0.38 \pm 0.021$) was significantly greater than

TABLE 4
ANALYSIS OF COVARIANCE: FIBRE DIAMETER ON BULB DIAMETER

Item	D.F.	M.S.
1. Overall regression	1	34,567***
2. Slope of means <i>v.</i> slope within groups†	1	2,498***
3. Scatter of means about regression of means	8	91***
4. Between slopes of group regressions	9	117***
5. Residual	606	26

† A group comprises the follicles examined from a phenotype.

*** Significant at $P < 0.001$.

the average coefficient within phenotypes ($b = +0.22 \pm 0.013$) indicating that a given increase in bulb diameter was associated with a greater increase in fibre diameter if the comparison was made between phenotypes than if it was made between follicles within a phenotype. The differences referred to here are thought to be biologically real, even though the validity of the statistical tests may be open to question because of the small but significant difference between the slopes of the individual regression lines (Table 4).

(iii) *Mitoses in Follicle Bulbs*.—Mitotic figures were confined to bulb tissue below the level of the tip of the papilla. Those occasionally seen in the papilla and the more frequent mitoses in the outer root sheath were not included in the present counts. Some of the cells produced in the follicle bulb pass into the follicle root sheaths and not into the fibre: no attempt was made here to estimate the ratio of cells that pass into the two channels. From qualitative observations it appeared likely that phenotypes did not differ in the proportion of cells that passed into the root sheaths. Therefore, the figures given for numbers of mitoses are probably a constant percentage overestimate of the actual number of cortical and fibre cuticle cells produced.

No obvious concentration of mitotic activity in any particular circumferential segment of the bulb tissue was observed, nor was there any evidence of a well-defined

germinal layer over the papilla, as implied by Rudall (1956, p. F-14) who referred to "the number of cells produced by the papilla surface".

Highly significant differences between phenotypes were observed in the mean number of mitoses per follicle bulb for each sample: phenotypes with larger bulb and fibre diameters had greater numbers of mitoses per bulb (Figs. 3 and 4; Tables 5 and 6). The correlations between mitoses¹ and bulb and fibre diameters in the three samples are given in Table 6. The correlations were substantially higher after colchicine than before (compare samples 2 and 3 with 1) in both comparisons. This is most likely due to the greater accuracy of mitotic counts following colchicine treatment. The association between mitotic activity and bulb or fibre diameter was studied therefore on samples 2 and 3.

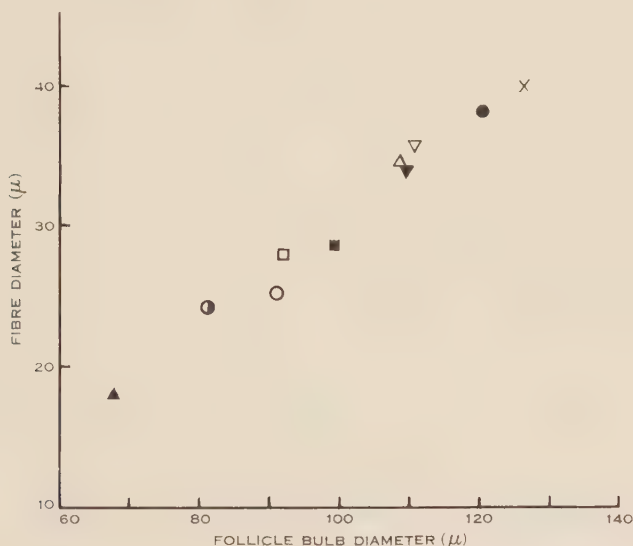


Fig. 2.—Follicle bulb diameters and fibre diameters—means of phenotypes examined:

- | | |
|-----------------------------------|---------------------------------------|
| ○ = B654 (strong-wool Merino) | △ = B434 (Corriedale, normal) |
| ◐ = E127 (fine-wool Merino) | ▽ = B434 (Corriedale, mosaic) |
| ● = B498 (carpet-wool Corriedale) | □ = B451 (medium-wool Merino, normal) |
| ▲ = A288 (Merino lustre mutant) | ▼ = B451 (medium-wool Merino mosaic) |
| ■ = SA4 (Southdown) | × = A961 (Border Leicester) |

(1) Bulb diameter and mitotic activity: The greater part of the variance in mitoses¹ was associated with variance in bulb diameter. This is clear from the correlation coefficients (Table 6) and from regression analyses (Table 7, item 1). Analysis of covariance indicated that the phenotypes differed significantly in the slope of the regression lines of mitoses¹ on bulb diameter in sample 2 but not in sample 3 (Table 7, item 4). This was largely due to a low regression coefficient in E127 (Merino fine wool) and when this sheep was omitted differences between the remaining coefficients were barely significant statistically. Apart from this, therefore, there was little indication that the major phenotypes examined here differed greatly in the relationship of mitotic activity to follicle bulb size. This implies that the increase

in the number of mitoses per unit time associated with an increase in follicle bulb diameter, is much the same in one phenotype as in another.

The regression coefficient of mitoses[‡] on bulb diameter were: sample 2—within phenotypes, $b = +0.026$, between phenotype means, $b = +0.041$; sample 3—within phenotypes, $b = +0.026$, between phenotype means, $b = +0.042$. In both samples the coefficient between phenotype means was significantly greater than the average coefficient within phenotypes. That is, there were more mitoses in phenotypes with larger bulbs than in phenotypes with small bulbs, even after adjustment for differences in bulb size.

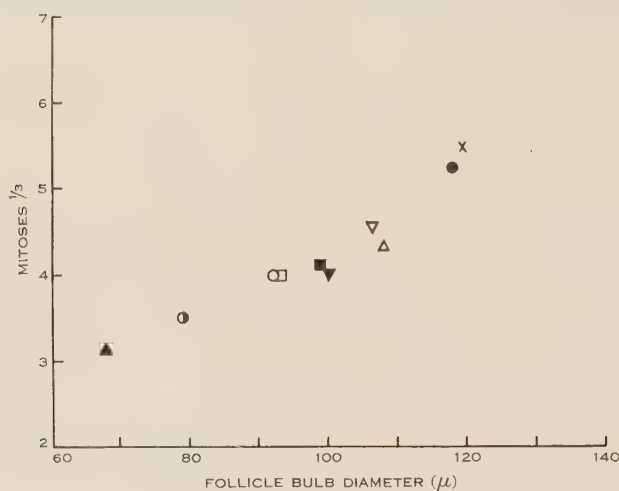


Fig. 3.—Mean mitoses[‡] and follicle bulb diameters of the phenotypes examined—sampled 2 hr after colchicine. Symbols as in Figure 2.

(2) Fibre diameter and mitotic activity: There was a close association between mitoses[‡] and fibre diameter both within phenotypes, and between means of phenotypes (Table 6; Table 7, item 1; and Fig. 4). However, there was a suggestion that the quantitative nature of this relationship differed slightly between phenotypes (Table 7, item 4, sample 3). Nevertheless, as the slopes of the regression lines of fibre diameter on mitoses[‡] for the individual phenotypes did not differ significantly in sample 2, it is concluded that the variance due to this factor was of little significance. The significant scatter of means about the regression of means indicates that after adjustment for differences in mitoses[‡], there were significant differences between phenotypes in the diameter of fibres produced (Table 7, item 3). These differences were relatively small (Fig. 4) but individuals tended to deviate from the regression of means in a consistent way between samples 2 and 3.

The problem arises of the relationship of the volume rate of fibre growth to mitotic activity. The only dimension of fibre growth actually measured was fibre diameter. The volume rate of fibre growth is determined by fibre length and fibre

cross-sectional area. In fibres of the same cross-sectional contour, area varies as the square of the diameter. An estimate of the relationship between mean fibre length and mean diameter of widely different phenotypes may be obtained from the results of Daly and Carter (1955) who worked with fine Merino, Polwarth, Corriedale, and Lincoln breeds. In their observations (their periods 2-10) an increase of 100% in fibre diameter was associated with an increase of *c.* 75% in fibre length. From the results of Oczan (1956) working with the same material it is possible to estimate the regression of length on diameter between fibres within sheep. This was some 30%

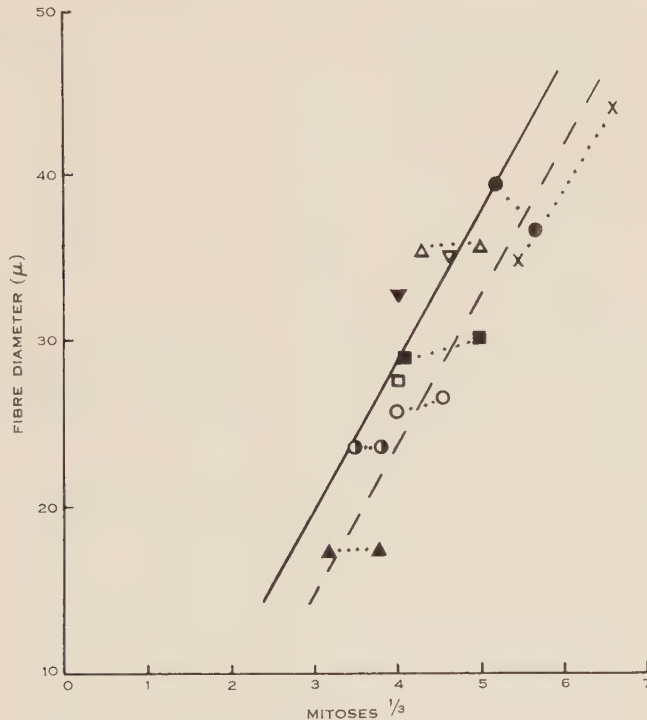


Fig. 4.—Mean fibre diameter and mitoses¹ of the phenotypes examined—sampled 2 and 3 hr after colchicine. Symbols as in Figure 2. The right-hand members of each pair linked by dotted lines are from samples taken 3 hr after colchicine; all others 2 hr after colchicine. Left and right regression lines refer to 2- and 3-hr samples respectively.

greater than that between breed means and hence 100% increase in diameter was associated with 100% increase in length. For the immediate purpose it may reasonably be assumed therefore that fibre length increases *pari passu* with fibre diameter when comparisons are made between fibres within a staple and between the means of major fleece types. In these comparisons fibre volume is therefore a linear function of the cube of the diameter.

Returning now to the problem of the relationship of volume rate of fibre growth to mitotic activity, this was examined on log transformed data of sample 2 by esti-

inating the gradient (g) of the log diameter : log mitotic number trend line. The gradient of this line is estimated from the ratio of the two standard deviations and, in situations where both variables are subject to error, gives a better estimate of the

TABLE 6
CORRELATION COEFFICIENTS BETWEEN BULB DIAMETER AND MITOSES[‡], AND BETWEEN FIBRE DIAMETER AND MITOSES[‡]

	Bulb Dia. <i>v.</i> Mitoses [‡]			Fibre Dia. <i>v.</i> Mitoses [‡]		
	Sample No. 1	Sample No. 2	Sample No. 3	Sample No. 1	Sample No. 2	Sample No. 3
Within phenotype	+0.52	+0.74	+0.71	+0.27	+0.67	+0.67
Between phenotypes	+0.40	+0.96	+0.97	+0.49	+0.87	+0.95
Total	+0.46	+0.85	+0.89	+0.40	+0.77	+0.87

quantitative relation between two related variables, than does the least squares regression line (Tessier 1948; Kermach and Haldane 1950). The slopes of the trend lines of log diameter on log mitotic number were: for total observations $g = +0.53$,

TABLE 7
ANALYSIS OF COVARIANCE: MITOSES[‡] ON BULB DIAMETER AND FIBRE DIAMETER ON MITOSES[‡]

Item	Bulb Dia. <i>v.</i> Mitoses [‡]				Mitoses [‡] <i>v.</i> Fibre Dia.			
	Sample 2		Sample 3		Sample 2		Sample 3	
	D.F.	M.S.	D.F.	M.S.	D.F.	M.S.	D.F.	M.S.
1. Overall regression	1	119.96***	1	160.11***	1	11,907.0***	1	13,026.0***
2. Slope of means <i>v.</i> slope within groups	1	5.79***	1	6.65***	1	1.0	1	2.7
3. Scatter of means about regression of means	8	8.72***	5	1.84***	8	281.0***	5	219.2***
4. Between slopes of group regression	9	2.96**	6	0.03(n.s.)	9	22.0	6	39.9*
5. Residual	216	0.128	156	0.163	216	26.7	156	17.8

* Significant at $P < 0.05$.

** Significant at $P < 0.01$.

*** Significant at $P < 0.001$.

within phenotypes $g = +0.55$, and between phenotype means $g = +0.50$. Clearly, there is little difference between the slopes of the means and that calculated within phenotypes and for the present purpose we may accept an average of $g = +0.50$.

Now, it has been shown above that in material of the type considered here, the volume of fibre output varies as the cube of the diameter: the gradient of the line relating log fibre volume to log mitotic number therefore will be 1.50 (i.e. 3×0.50). Thus an increase of 1 unit in log mitotic number (or 100% in mitotic number) was associated with an increase of 1.5 units in log fibre volume (or 150% in fibre volume). As fibre volume is determined by cell number and cell size, the additional 50% increase in volume above that due to number must have resulted from increase in cell size. A doubling of fibre volume in the present observations therefore was attributed about two-thirds ($1/1.5$) to an increase in cell number and one-third to

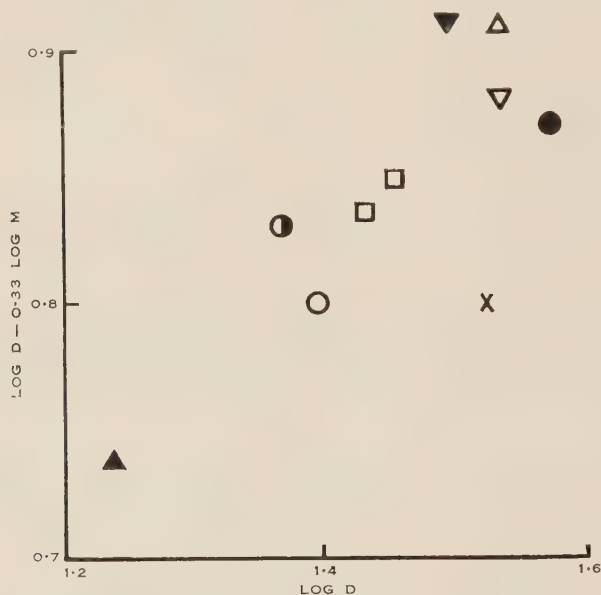


Fig. 5.—Mean cell linear index ($\log D - 0.33 \log M$) and $\log D$ of the phenotypes examined. Symbols as in Figure 2.

cell volume. Deviations from this average relationship also occurred. In the relationship between mitoses³ and fibre diameter illustrated in Figure 4, points lying above the regression lines are those in which fibre diameter is greater, for a given mitotic rate, than would be expected. This could arise from increased cortical cell size. Similarly, the reverse situation would hold for points below the line.

Cell volume was not measured but an index of cell linear dimension is given by fibre diameter \div mitoses³. The correlation coefficients between this index (in log units) and mitotic number (in log units) were: between phenotype means, $r = +0.44$, within phenotypes $r = +0.06$; total data, $r = +0.24$. Only the last coefficient was statistically significant.

In Figure 5 the index of cell linear dimension (in log units) is plotted against log fibre diameter. Increasing cell linear index was generally associated with increasing fibre diameter. The correlation coefficients were: between fibres within phenotypes, $r = +0.80$ and between phenotype means, $r = +0.80$. Although this

analysis has inherent limitations because one variable has been mathematically derived from the other, these errors are considered insufficient to negate the general conclusion that larger fibres are generally comprised of larger cortical cells.

IV. DISCUSSION

(a) *The Proliferative Phase of Fibre Growth*

The present results demonstrate the importance of the number of cells produced in the follicle bulb as a determinant of fibre diameter and of the estimated volume rate of fibre growth, both between follicles in a given phenotype and between widely different phenotypes. In this respect, measurement of mitotic activity becomes a most useful test system for the study of factors controlling fibre growth.

It is reasonable to conclude that the relationship of mitoses to fibre growth is one of cause and effect in a metrical sense because of the structural relationship between them. On the assumption that the volume rate of fibre growth is proportional to the cube of the diameter, it was estimated that on the average about two-thirds of the increase in fibre volume between small and large fibres was due to an increase in cell number and one-third to an increase in cell size. If the volume rate of growth was a function of less than the third power of diameter, then the relative significance of cell number would be greater and that of cell size correspondingly less. If the rate of fibre growth was a function of the second power of diameter (i.e. cross-sectional area), then, in the present observations, fibre output would be wholly a function of cell number. The conclusion that at least a portion of the differences in the volume of fibres is due to difference in the size of the component cortical cells receives support from the work of Appleyard and Dymoke (personal communication, 1960) who observed a positive correlation between cortical cell diameter and fibre diameter. On the other hand, the finding that differences in cell number are of greater significance than differences in cell size is very much to be expected from general considerations of the biology of tissue and cell growth and of cell division: cells tend to be relatively constant in size; they will not divide until they have attained about 80% of normal size; nor will they pass a certain limiting size (Swann 1958; von Bertalanffy 1960).

Because the rate of fibre output largely depends on the number of cells produced in the bulb tissue, this does not necessarily mean that the follicle papilla is not a significant organ in fibre growth. The importance of dermal tissue in differentiation in feather growth (Lillie and Wang 1941) and mammalian follicle development (Hardy and Lyne 1956) is clearly established. In these well-studied situations, the action of the dermal component as an inductor of epidermal activity appears to be of the permissive or threshold kind. There is no evidence that variations in the production of some hypothetical inductor substance(s) cause quantitative variation in the activity of the reacting tissue. It is more likely that variable activity in the reacting tissue is a consequence of other stimuli, e.g. hormones, metabolites, etc. A correlation between papilla volume and fibre growth (Rudall 1956) does not establish cause and effect, but may merely reflect a close allometric relationship between the size of the papilla and the size of the bulb, which largely envelopes it.

In this respect, I do not agree with Rudall (1956) that "it is fairly certain" that "the papilla . . . is the chief determiner of the characteristics of the fibre produced". There is at present insufficient evidence to justify this statement.

In the present observations, the differences in number of cells produced in follicle bulbs per unit time must have arisen mainly from differences in the number of germinal cells rather than from differences in the cell turnover time. Individual bulb diameters ranged over a factor of 3·8: this is equivalent to a factor of *c.* 55 in bulb volume. While in extreme cases, cell volumes may vary by a factor of 6–8 (Thompson 1942) it is more likely in the present material that the factor was less than 3·5. No systematic study of bulb cell size was made but it is considered that an increase of 50% in cell diameter (350% in cell volume) would be visibly obvious. No such increases were found and it is concluded therefore that the differences in bulb volume were very largely due to differences in the number of cells present and not to differences in cell size.

The relative significance of mitotic rate and of cortical cell size as determinants of the volume of fibre is also of interest in the problem of utilization of food nutrients. It is possible that the production of a given volume of fibre from a relatively few large cells rather than from many small ones would be more efficient from the energetic aspect, if the view of Bullough (1952), that mitosis is a relatively expensive process in terms of energy, is correct.

(b) The Follicle as an Organ of Protein Synthesis

In the broadest sense, the production of a fibre is a two-stage process: firstly the proliferative phase involving cell reproduction in the follicle bulb, and secondly, the keratinization phase, involving the physicochemical transformation of the plastic, cellular mass produced from the bulb into keratinized fibre of low moisture content. There is at present little really satisfactory evidence of the site(s) at which synthesis takes place and particularly of the relative importance of the follicle bulb (proliferative phase) and the lower follicle (keratinization phase). Radioautographic studies using ³⁵S-cystine suggest that much of this amino acid is incorporated in the fibre above the bulb and in the prekeratinization and keratinization zones (Bern 1954; Bern, Harkness, and Blair 1955; Ryder 1956). Mercer (1949) was unable to detect the addition of significant quantities of matter above the bulb, although his technique (simple drying) may not have been particularly sensitive. Harkness and Bern (1957), however, noted low radioactivity in the keratogenous zone of the follicles of rats given ¹⁴C-protein hydrolysate, suggesting the possibility that other new material may also be incorporated here. It seems likely that by far the greater part of the synthetic processes takes place in the bulb tissue. Assuming it to be a sphere, the weight of a follicle bulb, including the papilla, of 100 μ diameter would be about 0·5 μ g. This would probably contain *c.* 0·1 μ g of dry tissue and would produce fibre at the rate of about 0·3 μ g per day under favourable nutritional conditions. This involves a turnover time of the order of 8 hr, and suggests that the follicle bulb in sheep is one of the most active sites of protein synthesis in mammals. An approximate check on this estimate may be obtained from the turnover time of the cell population. Comprehensive counts of the total number of cells in the follicle bulb have not yet

been attempted, but preliminary observations on bulbs about 100 μ diameter indicate the number to be in the order of 700–1000. If mitotic activity in the bulb is relatively uniform throughout the day, and Bullough and Laurence (1958) have provided evidence that this is so in mice, then from the present observations 850 cells would be produced by a bulb 100 μ diameter in *c.* 20 hr. This figure is to be compared with the 8 hr arrived at above. The agreement is considered good in view of the assumptions involved.

From the large literature on the physical properties and chemical composition of wool fibres two broad conclusions emerge. Firstly, wool fibres differ greatly in dimensional characteristics: such differences may be the consequence of both genetic and nutritional factors. Secondly, with one exception to be discussed below, the chemical composition and physical properties of fibres are remarkably resistant to changes in the nutritional environment, or of the genetic composition of the animals which produce them. Both of these factors can cause very great variation (factors of 5–10 \times) in dimensional characteristics. This failure to find "effects" of these factors on the composition of fibres could of course arise simply because those features in which differences do occur have not yet been discovered. In this respect the finding that nutrition does affect plasticity of wool fibres may be significant (Whiteley and Speakman 1959). However, from the results reported here, and from the effects of nutrition on mitotic activity in the epidermis (Bullough and Laurence 1958) it is concluded that variations in the volume rate of fibre synthesis (*i.e.* dimensional characteristics) are very largely a function of the proliferative phase of growth. Further, there appears to be only one naturally occurring situation in which the keratinization phase is seriously disturbed, *viz.* copper deficiency. In this condition, severe deficiency causes a serious delay in the process of keratinization and altered physical and chemical properties (Marston 1946), but only a relatively small reduction in the rate of fibre output (Marston and Lee 1948). This suggests that keratinization is not an important rate-limiting process with respect to the volume of fibre growth while it is certain that the proliferative phase in the bulb is such a rate-limiting process.

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CHEMICAL CHANGES IN WOOL TREATED WITH SOLUTIONS OF IODINE

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Summary

The chemical changes in wool associated with iodination in various solvents have been investigated. In many solvents a large proportion of the tyrosine residues is iodinated but in others this modification is negligible. Oxidation of disulphide bonds occurs in all solvents. This appears to proceed stepwise and intermediate oxidation products of cystine residues are formed in considerable amounts during iodination in solvents such as ethanol. Oxidation of sulphydryl groups and ethylation of carboxyl groups also occur in this solvent. The presence of small amounts of water in the ethanol increases the rate of reaction with the wool. Iodination in ethanol also causes a considerable decrease in solubility in solutions of urea-bisulphite, and an increase in trypsin digestibility and alkali solubility.

The results are discussed in relation to recent suggestions that the tyrosine residues of wool are confined to the amorphous regions of the fibre (Ghosh, Holker, and Speakman 1958) and that tyrosine residues which react with iodine in propanolic solutions are in the non-crystalline portions of the fibre (Haly and Feughelman 1960). Factors affecting the solubility of wool in urea-bisulphite solutions are also discussed.

I. INTRODUCTION

Although iodine has been used in protein research as a more or less specific reagent for tyrosine, the complexity of the reaction of iodine with proteins is now being more widely recognized (Ramachandran 1956). Apart from substitution reactions with the tyrosine and histidine residues of proteins, oxidation of tryptophan, serine, threonine, methionine, cysteine, and cystine residues has been demonstrated. By analogy with the action of perbenzoic acid and hydrogen peroxide on cystine (Lavine 1936), Ramachandran (1956) suggests that partial oxidation products of cystine residues of proteins may be formed during iodination. Some indication that such products are formed during the treatment of wool with bromine (Consden, Gordon, and Martin 1946) or with hydrogen peroxide (Consden and Gordon 1950) has already been obtained. More definite evidence for the formation of such partial oxidation products of cystine during oxidation of wool with peracetic acid has now been provided by Maclaren, Leach, and O'Donnell (1959).

Earlier studies of the iodination of wool have been concerned primarily with locating tyrosine residues in wool in radioautographic experiments (Richards and Speakman 1955), in X-ray diffraction studies (Fraser and MacRae 1957), or for assessing the accessibility of tyrosine residues to certain solvents (Harrison and Speakman 1958; Ghosh, Holker, and Speakman 1958; Haly and Feughelman 1960). Similarly Haly, Feughelman, and Griffith (1957) have related changes in the supercontraction behaviour of wool fibres following iodination to substitution in the tyrosine residues of wool.

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In order to interpret the effects of iodination on the physical and chemical properties of wool we found it necessary to obtain more complete information concerning the reactions of iodine with wool particularly with respect to iodination in ethanol. Richards and Speakman (1955) have already reported that the histidine and tryptophan residues of wool are not modified by iodination in ethanol whereas the tyrosine residues are almost quantitatively converted to di-iodotyrosine residues. However, we considered three additional types of reaction were likely to occur: oxidation of sulphhydryl or disulphide groups, ethylation of carboxyl groups catalysed by HI formed in oxidation and substitution reactions, and changes in protein configuration which would be facilitated by rupture of disulphide bonds. Particular attention has been given to these reactions during iodination in aqueous or anhydrous ethanol but comparative studies have been made with other solvents.

Earlier studies on the arrangement of tyrosine residues in the wool fibre and their importance in determining the physical properties of wool are discussed in the light of the additional chemical data concerning iodination.

II. MATERIALS AND METHODS

The Corriedale 56's wool used in these experiments was cleaned by extraction three times with light petroleum, twice with cold ethanol, and several times with cold distilled water. It was dried in a stream of air at 40°C.

Unless otherwise specified non-aqueous solvents were dried and redistilled. Alcohols were dried by the method of Manske (1931), other solvents by shaking with the appropriate dessicant. For use with these solvent systems the wool was dried *in vacuo* at 40°C over P_2O_5 for 3 days. All other reagents were of "Analar" quality.

With each solvent system iodination of the wool was carried out at 20°C for periods which varied according to the rate of iodination. A liquor ratio of 100 : 1 was used for all solvents. The subsequent procedure was similar to that described by Richards and Speakman (1955) and by Harrison and Speakman (1958): thorough rinsing with clean solvent, soaking twice in fresh solvent for periods of 30 min (this was followed by rinsing in ethanol if the solvent was non-miscible with water), soaking for 24 hr in 0.1N $Na_2S_2O_3$ containing 0.2 g/l Na_2CO_3 , and soaking with many changes of distilled water until the wool was free of iodide (12–14 days). The wool was then air dried and conditioned.

The tyrosine contents of treated and untreated wool samples were determined by a modification of the method of Bernhart (1938) previously described (Crewther and Dowling 1960). Approximately 14% of the tyrosine is destroyed during hydrolysis in alkali in the presence of the other amino acids of wool, and the results obtained have accordingly been corrected for this loss. The disulphide plus sulphhydryl ($-SS-$ + $-SH$) contents of the wool samples were estimated both by the methods of Shinohara (1936) using acid hydrolysates, and by the polarographic method of Leach (1960) using intact fibres. Sulphydryl contents were estimated either by the method of Leach (1960) or that of Burley (1956). The values quoted in this paper were obtained by the former method but similar results were obtained by the latter.

Primary amino groups in intact wool were determined by a method similar to that of McPhee (1958), the alkali solubility by the method of Harris and Smith (1936), the solubility in urea-bisulphite solution by the method of Lees and Elsworth (1956) and the solubility in alkaline solutions of thioglycollate by the method of Lennox (1956). The trypsin digestibility of the wool samples was determined following pretreatment in buffers at pH 4 and pH 11 as described by Crewther and Pressley (1958). The iodine content of treated wool samples was determined by the method described by Richards and Speakman (1955).

TABLE 1
COMPARISON OF THE EFFECTS OF IODINATION IN DIFFERENT SOLVENTS ON THE CONTENTS OF
TYROSINE AND $(-SS- + -SH)$ OF WOOL

Iodination Treatment	Time of Iodination (days)	Tyrosine (μ moles/g)	$(-SS- + -SH)^*$ (μ moles/g)	(T/S) †
Untreated	—	307	505	
0.78N I ₂ /ethanol	0.25	151	418	3.33
0.78N I ₂ /ethanol	3	49	298	2.05
0.78N I ₂ /propanol	6	236	450	2.10
0.78N I ₂ /propanol	15	206	428	2.15
0.78N I ₂ /acetone	7	309	482	0.00
0.78N I ₂ /acetone	15	310	444	0.00
0.33N I ₂ /CCl ₄	15	277	428	0.65
0.33N I ₂ /CH ₂ Cl ₂	3	243	469	2.92
0.33N I ₂ /CH ₂ Cl ₂	15	126	428	3.92
0.078N I ₂ /0.1N KI, unbuffered	0.25	144	369	1.97
0.078N I ₂ /2N HCl + c. 0.1N KI	0.25	295	351	0.12
0.078N I ₂ /0.1N KI in 0.1M sodium borate at pH 9.0	0.25	120	415	3.43

* Determined on intact wool by polarographic methods.

† T = percentage decrease in tyrosine content, S = percentage decrease in $(-SS- + -SH)$.

III. RESULTS

(a) *Effects of Iodination in Various Solvents on the Tyrosine and $(-SS- + -SH)$ Contents of Wool*

Table 1 lists the tyrosine contents and $(-SS- + -SH)$ contents of wool samples which had been treated with various solutions of iodine. With ethanol, propanol, and acetone, 0.78N iodine was used to permit comparison within the series and with the results of Richards and Speakman (1955) and Harrison and Speakman (1958). With carbon tetrachloride and methylene dichloride the concentration (0.33N) corresponds with saturation of the former solvent at room temperature. The very rapid reaction of iodine with wool in aqueous media led to the use of more dilute solutions of iodine in aqueous KI.

In all solvents a considerable proportion of the disulphide groups of the wool was oxidized. There was no obvious correlation between the loss of tyrosine and the decrease in $(-SS- + -SH)$. Whereas prolonged treatment in I_2/CH_2Cl_2 caused iodination of about 60% of the tyrosine residues and only 15% decrease in $(-SS- + -SH)$, treatment in I_2 /acetone caused no measurable loss of tyrosine but a decrease of about 12% in the content of $(-SS- + -SH)$. In aqueous KI the pH of the solutions influenced the relative effects on tyrosine residues and on $(-SS- + -SH)$.

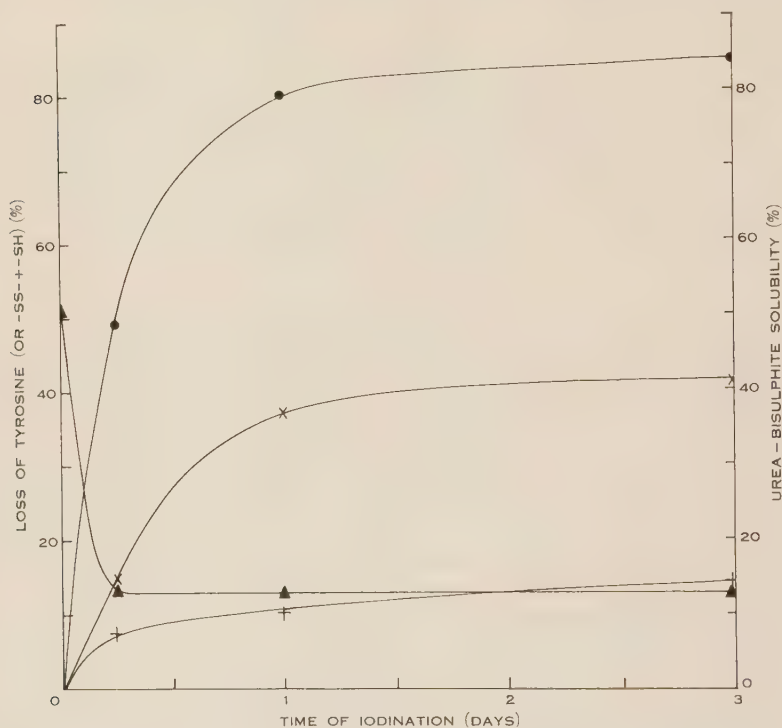


Fig. 1.—Variations in the tyrosine content, the $(-SS- + -SH)$ content as determined by the methods of Shinohara and Leach, and the urea-bisulphite solubility of wool with time of treatment in anhydrous ethanolic iodine solution. ● Loss of tyrosine. × Loss of $(-SS- + -SH)$ (Leach). + Loss of $(-SS- + -SH)$ (Shinohara). ▲ Urea-bisulphite solubility.

Iodine determinations were carried out on samples of wool which had been iodinated in anhydrous ethanol for 72 hr and in anhydrous propanol for 15 days. The values obtained corresponded with conversion of 91 and 37% respectively of the tyrosine residues to di-iodotyrosine residues. As estimated by decrease in tyrosine content the values were 84 and 33% respectively (Table 1). The rates of reaction of wool with iodine in anhydrous ethanol, anhydrous propanol, and unbuffered aqueous KI are shown in Figures 1-3. The maximum decreases in tyrosine content during iodination in ethanol, propanol, and aqueous KI were 84, 33, and 50% even with prolonged reaction.

(b) Oxidation of Sulphydryl and Disulphide Groups

Table 2 shows that the sulphydryl content of wool was decreased by all of the iodination treatments investigated but none completely removed the sulphydryl groups (cf. Leveau 1959). The values obtained by the method of Burley were similar to those reported in Table 2 but the values obtained by both methods for iodinated wool samples lacked precision.

Table 1 indicates the extensive oxidation of disulphide groups which occurs during iodination in certain solvents and Table 3 compares the values for the $(-SS- + -SH)$ contents obtained by the method of Leach using intact wool and that of Shinohara using wool hydrolysates. Wool samples which had been iodinated in ethanol

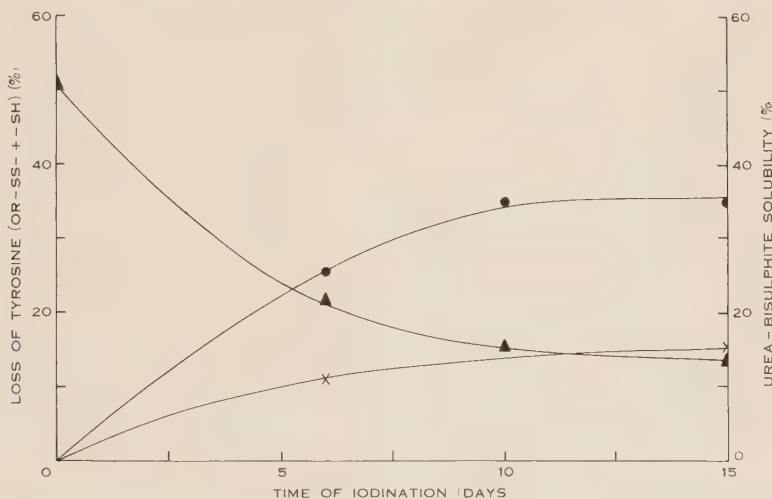


Fig. 2.—Variations in the tyrosine content, the $(-SS- + -SH)$ content as determined by the method of Leach, and the urea-bisulphite solubility of wool with time of treatment in anhydrous propanolic iodine solution. ● Loss of tyrosine. × Loss of $(-SS- + -SH)$ (Leach). ▲ Urea-bisulphite solubility.

or ethanol/water mixtures (Table 3) were treated with 2N HCl/2M KI solutions at 20°C overnight as described by Harris and Smith (1937). Considerable amounts of iodine were liberated and the $(-SS- + -SH)$ content increased in some samples by more than 100%. Treatment of the wool samples with 2N HCl containing no KI did not release iodine although the $(-SS- + -SH)$ contents increased.

(c) Effects of Water on Iodination in Alcohols

Table 3 and Figure 4 show that the presence of water in the ethanol used as solvent increased the rate of iodination of tyrosine residues and the rate of oxidation. There was also an increase in the extent of these reactions. The reaction in propanol was affected to an even greater extent by the presence of water (Table 4). The rate of reaction was greatly increased and there was a major increase in the extent of reaction.

(d) *Ethylation of Carboxyl Groups*

Circles of plain-weave Merino 64's fabric, each weighing 11 mg, were treated for 72 hr at 20°C with 0.78*N* iodine in anhydrous ethanol containing [1^{14}C]ethanol. The iodinated samples were washed twice in the radioactive solvent for 30-min periods, then rinsed with water, treated with $\text{Na}_2\text{S}_2\text{O}_3$ solution, and washed thoroughly in the usual manner. For comparison, similar fabric circles were treated in the same solvent containing no iodine but with the addition of concentrated HCl to give a final concentration of 0.1*N*. The ethylation process catalysed by the HCl was complete in about 35 days. After complete removal of free ethanol, chemical analysis showed that esterification of the carboxyl groups amounted to 480 $\mu\text{-equiv/g}$.

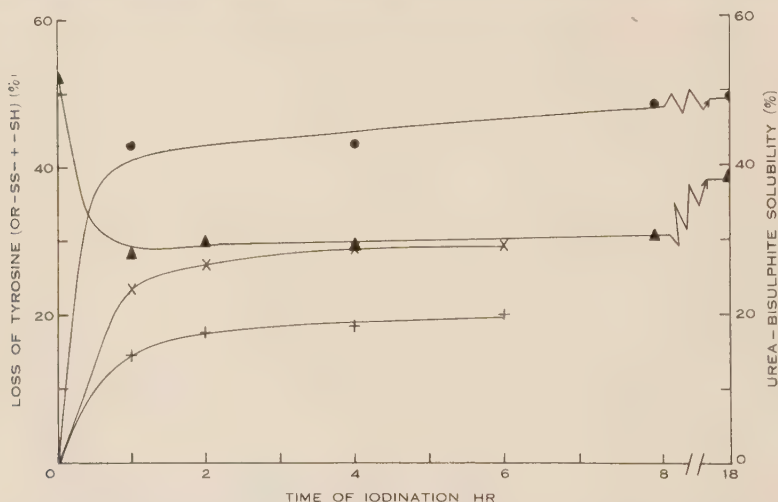


Fig. 3.—Variations in the tyrosine content, the $(-\text{SS}- + -\text{SH})$ content as determined by the methods of Shinohara and Leach, and the urea-bisulphite solubility of wool with time of treatment in aqueous KI_3 . ● Loss of tyrosine. × Loss of $(-\text{SS}- + -\text{SH})$ (Leach). + Loss of $(-\text{SS}- + -\text{SH})$ (Shinohara). ▲ Urea-bisulphite solubility.

The radioactive count of this material was compared with that of the iodinated sample, also carefully freed of ethanol, and the estimate of ethoxy groups in the wool obtained in this way was 298 $\mu\text{-equiv/g}$. Further repeated washing of the iodinated sample in water and acetone did not decrease the count. Direct chemical determination of ethoxy content gave a value of 192 $\mu\text{-equiv/g}$.

(e) *Trypsin Digestibility*

Wool which had been iodinated in undried ethanol for 3 days was digested by solutions of crude trypsin even after soaking at 30°C for 20 hr at pH 4 (Table 5). The original wool after similar soaking did not lose weight during digestion. Pre-soaking at pH 11 resulted in very extensive digestion of the iodinated wool and, as previously reported (Crewther 1956), a small loss of weight in the uniodinated wool. The effect was much less if anhydrous ethanol was used as solvent during

iodination and with anhydrous propanol or unbuffered aqueous KI as solvents the digestibility was very small.

(f) *Solubility and other Data*

Iodination of wool in ethanol or propanol causes the solubility in urea-bisulphite solution to fall from about 50% to about 13% (Table 6). This occurs rapidly in ethanol but much more slowly in propanol (Figs. 1 and 2). With unbuffered aqueous KI as solvent the urea-bisulphite solubility falls to a minimum even more quickly than in ethanol, and then slowly increases (Fig. 3). Iodination in ethanol causes a similar

TABLE 2
EFFECTS OF IODINATION IN VARIOUS SOLVENTS ON THE
SULPHYDRYL CONTENT OF WOOL
Sulphydryl content determined by the method of Leach (1960)

Iodination Treatment	Time of Iodination	Sulphydryl Content (μ moles/g)
Untreated	—	25.1
0.78N I ₂ /ethanol	6 hr	13.9
0.78N I ₂ /ethanol	72 hr	13.6
0.78N I ₂ /ethanol, 10% water	6 hr	10.9
0.78N I ₂ /propanol	15 days	19.5
0.078N I ₂ /0.1N KI, pH 6	6 hr	19.5
0.078N I ₂ /0.1N KI, pH 9	6 hr	14.6

decrease in solubility in alkaline thioglycollate solutions but iodination in unbuffered aqueous KI has no measurable effect on the solubility in this reagent (Table 6). The alkali solubility of wool is increased to some extent by iodination in ethanol (Table 6) and there is also a very small increase in primary amino groups from 190 to 202 μ -equiv/g.

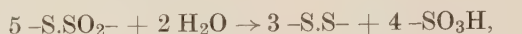
IV. DISCUSSION

(a) *Chemical Changes Caused by Iodination of Wool*

Apart from the iodination of tyrosine residues, the most important reaction of iodine with wool appears to be with the disulphide residues (Tables 1 and 3). The discrepancies between the estimates of ($-\text{SS}- + -\text{SH}$) by the method of Shinohara, using acid hydrolysates of the wool samples, and that of Leach, using intact fibres (Table 3; Figs. 1 and 3), indicate the presence of intermediate oxidation products of disulphide groups. This is confirmed by the increase in the values obtained by the latter method and the release of free iodine after treating the fibres with HCl/KI.

Maclaren, Leach, and Swan (1960) have shown that both "cysteine sulphinic acid" and the thiolsulphonate derived from cystine undergo disproportionation

during acid hydrolysis to produce cystine, cysteic acid, and possibly small amounts of other products. Each mole of the thiolsulphonate produced 0.52 mole of cystine, each mole of the sulphinic acid produced 0.16 mole of cystine. The former result most closely corresponds with the equation



the theoretical yield of disulphide per mole of $\text{--S.SO}_2\text{--}$ being 0.6 mole. The yield of disulphide from the thiolsulphinatate group would be proportionately greater than 0.52 (theoretical 0.8) and that from more oxidized intermediates lower than 0.52 mole.

TABLE 3
COMPARISON OF THE ($\text{--SS--} + \text{--SH}$) CONTENT OF ACID HYDROLYSATES AND UNHYDRO-
LYSED SAMPLES OF IODINATED WOOL

Iodination Treatment	Time of Iodination	($\text{--SS--} + \text{--SH}$) Content ($\mu\text{moles/g}$) of:	
		Hydrolysate (Shinohara)	Intact Fibre (Leach)
Untreated	—	519	504
0.078N $\text{I}_2/0$, 1N KI	6 hr	407	366
0.33N I_2/CCl_4	15 days	505	427
0.33N $\text{I}_2/\text{CH}_2\text{Cl}_2$	3 days	487	465
0.78N $\text{I}_2/\text{acetone}$	15 days	472	460
0.78N $\text{I}_2/\text{ethanol}$	72 hr	419	290
0.78N $\text{I}_2/\text{ethanol}$, 0.1% water	72 hr	421	254
0.78N $\text{I}_2/\text{ethanol}$, 1.0% water	72 hr	382	219
0.78N $\text{I}_2/\text{ethanol}$, 10% water	72 hr	262	106

Because of this disproportionation in acidic or alkaline solutions it is not possible to determine the cysteic acid content of the intact wool nor is a direct determination of the thiolsulphinatate or thiolsulphonate content possible. The ratio $(\text{H} - \text{I})/(\text{I}_0 - \text{I})$, where H = the disulphide content of the hydrolysate, I = the disulphide content of the intact iodinated fibre, and I_0 = the disulphide content of untreated wool, provides an estimate of the molar yield of disulphide produced during hydrolysis. The values so obtained for iodination in anhydrous ethanol, and ethanol containing 0.1, 1.0, and 10% water are 0.65, 0.71, 0.60, and 0.41 respectively. These figures represent minimum values since the formation of cysteic acid during iodination would give a correspondingly low value of this ratio. This indicates that the thiolsulphinatate group is one of the intermediates in the oxidation of the disulphide bonds. Iodination in CCl_4 or CH_2Cl_2 also gives rise to considerable amounts of oxidation intermediates whereas oxidation in aqueous KI or acetone proceeds largely to completion (Table 3).

Little is known of the nature of the reaction of sulphhydryl groups of the wool with iodine. Some sulphenyl iodide groups may be formed (Fraenkel-Conrat 1955; Cunningham and Nuenke 1959) but as Fraenkel-Conrat has found that such groups are very reactive it is improbable that they would survive the treatment with thiosulphate solution. Sulphhydryl groups would probably be oxidized to the disulphide (Fraenkel-Conrat 1955) if their position in the protein permitted this reaction but some may also be converted to sulphonic acid groups.

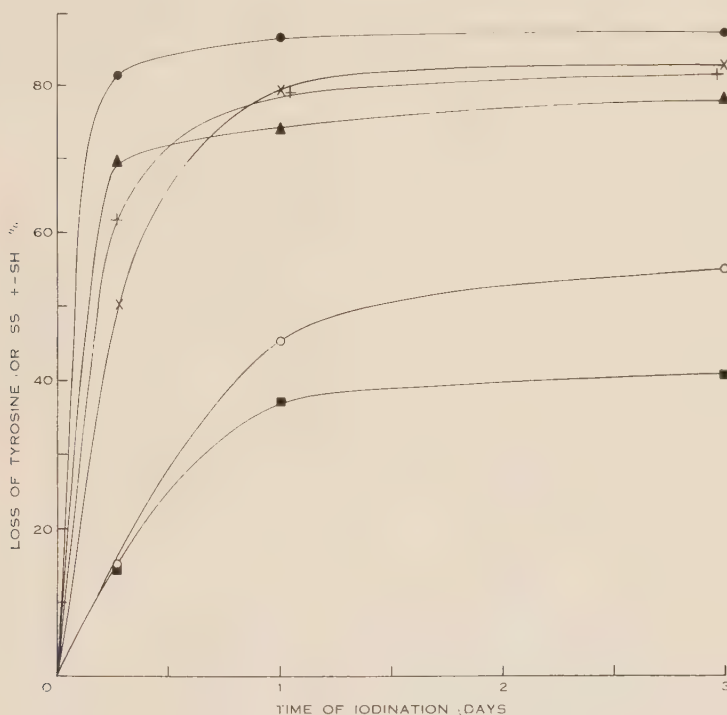


Fig. 4.—Effect of water on the reaction of the tyrosine residues and disulphide groups of wool with iodine in ethanolic solution. (—SS— + —SH) contents determined by the method of Leach.

Loss of Tyrosine	Loss of (—SS— + —SH)
× Anhydrous ethanol	■ Anhydrous ethanol
+ 1% aqueous ethanol	○ 1% aqueous ethanol
● 10% aqueous ethanol	▲ 10% aqueous ethanol

The values obtained for the ethoxy contents of wool fabric after iodination in ethanol leave no doubt that some 200 μ -equiv. of carboxyl groups per gram of wool were esterified. The discrepancy between the value obtained by direct analysis and that obtained by the radiotracer technique is attributable to the dimensional instability of the fabric which results in high values. This technique serves merely to confirm the presence of considerable amounts of ethoxy groups in the wool. Under similar conditions of time and temperature in ethanol containing 0.1N HCl

the ethoxy content of the fabric reaches only about 100 μ -equiv/g (Springell, personal communication). This unexpectedly high value for esterification cannot be accounted for completely in terms of the production of HI during oxidation and substitution; these reactions would give a final concentration of about 0.02N HI in the liquor.

(b) *The Urea-Bisulphite Solubility of Iodinated Wool*

The decrease in solubility in this reagent observed when wool has been subjected to various chemical or physical treatments has been attributed to formation of lanthionine (Lees and Elsworth 1956), to sulphydryl-disulphide interchange (Kessler and Zahn 1958), and to configurational changes in proteins (Swan 1959). Leveau

TABLE 4
EFFECT OF WATER ON THE IODINATION OF WOOL IN PROPANOL
Iodination was at 20°C

Solvent for Iodination	Time of Iodination (days)	Tyrosine Content (μ moles/g)	Solvent for Iodination	Time of Iodination (days)	Tyrosine Content (μ moles/g)
Untreated	—	307	Propanol + 1.0% water	3	221
Anhydrous propanol	3	283	Propanol + 1.0% water	6	159
Anhydrous propanol	6	227	Propanol + 10% water	3	42
			Propanol + 10% water	6	35

(1959) has already reported a decrease in urea-bisulphite solubility when wool is iodinated in ethanol and attributes this to strengthening of the hydrogen bonds between tyrosine side-chains and ionized carboxyl groups. This explanation predicts a direct relationship between solubility and the extent of iodination of tyrosine residues in various solvents. No such relationship has been observed (Tables 1 and 6; Fig. 3). In addition this explanation cannot account for the decrease in solubility in alkaline thioglycollate solution (Table 6) which results from iodination in ethanol. Under the conditions of this test the di-iodotyrosine residues would be completely ionized and therefore incapable of hydrogen bonding with ionized carboxyl groups.

Unfortunately the unlikely possibility that lanthionine is formed during the iodination cannot be checked since partial oxidation products of cystine produce small amounts of lanthionine during acid hydrolysis (Maclaren, unpublished results). The other alternatives are equally improbable. Although the wool proteins appear to have been disordered by iodination in ethanol containing small amounts of water this effect is insignificant in anhydrous ethanol (Table 5); yet the solubility in urea-bisulphite solution decreases to much the same value whether the solvent is anhydrous or not. Hence there is no reason to attribute insolubility to configurational changes in the wool proteins. Likewise sulphydryl-disulphide interchange is inhibited by oxidation. The solubility of the wool proteins is known to depend largely on

their net negative charge (O'Donnell 1954; Gillespie 1956). This would be influenced by oxidation of disulphide groups, ethylation of carboxyl groups, iodination of tyrosine groups, and the final pH of the urea solutions. Changes in solubility cannot therefore be attributed to a single chemical reaction of iodine with the wool.

(c) *Accessibility of Tyrosine Residues in Wool*

Table 1 shows that the substitution of tyrosine residues and the oxidation of disulphide groups proceed at different relative rates in different solvents. The use of this type of data as an index of the ability of solvents to diffuse into wool (Harrison and Speakman 1958) is therefore open to doubt since, for example, different conclusions would be drawn according as the iodination of tyrosine or the oxidation of disulphide was taken as the index of accessibility of the fibre.

TABLE 5
TRYPSIN DIGESTIBILITY OF IODINATED WOOL

Iodination Treatment	Time of Iodination	pH of Pretreatment	Digestibility* (%)
Untreated	—	4.0	-1.0
		11.0	1.5
0.78N I ₂ /ethanol (undried)	1 hr	4.0	1.0
0.78N I ₂ /ethanol (undried)	72 hr	4.0	23.8
		11.0	53.8
0.78N I ₂ /ethanol (anhydrous)	72 hr	4.0	-0.1
		11.0	16.0
0.78N I ₂ /propanol	15 days	4.0	-1.2
0.078N I ₂ /KI	6 hr	4.0	-0.3
		11.0	1.8

* Negative values indicate a gain in weight after treatment with the enzyme. This probably results from adsorption of the enzyme.

The incomplete iodination of tyrosine residues in the various solvents may be the result of (1) very slow reaction of the residue with iodine, (2) the establishment of an equilibrium, or (3) the inaccessibility of part or all of the tyrosine to the reagent due to its incorporation in an ordered structure or because of steric effects of other residues. With aqueous KI₃, I₂/ethanol, and I₂/propanol, constant levels of iodination of tyrosine residues were reached but in each case the extent of iodination was different. If a difference in accessibility of crystalline and non-crystalline regions of the fibre were the factor chiefly responsible for determining the final level of iodination, similar levels of iodination would be expected in all solvents. The results suggest that in these solvents the establishment of an equilibrium is responsible for limiting the extent of iodination of tyrosine residues; this applies particularly with solutions of I₂ in aqueous KI where changes in pH influence the final extent of iodination (Table 1). The effects of small amounts of added water on the extent of iodination in ethanol and propanol (Tables 3 and 4) support this view.

The results have a bearing on the conclusions of Ghosh, Holker, and Speakman (1958) and those of Haly and Feughelman (1960) regarding the location of tyrosine residues in the wool fibre. The former authors conclude that the tyrosine residues of wool are located in the "amorphous regions" of the wool fibre and use the accessibility of 96% of the tyrosine residues of wool to iodine in ethanol (not anhydrous) as evidence for this assumption. Haly and Feughelman, on the other hand, suggest that the partial iodination of tyrosine in propanol/I₂, taken in conjunction with the absence of 33 Å repeat on the X-ray diffraction pattern, is evidence that only tyrosine residues in the matrix or less ordered regions are iodinated in this solvent.

The conclusion of Ghosh, Holker, and Speakman would be valid only if it were known that the crystalline regions of the fibre are too closely packed to allow ingress of iodine and that the reaction with iodine does not cause configurational changes in the wool proteins, particularly in the crystalline regions.

TABLE 6
EFFECTS OF IODINATION OF WOOL ON ITS SOLUBILITY IN STANDARD REAGENTS

Iodination Treatment	Time of Iodination	Urea-Bisulphite Solubility (%)	Alkaline Thioglycollate Solubility (%)	Alkali Solubility (%)
Untreated	—	51	39	8.5
Ethanol/I ₂	3 days	13	20	13.2
Propanol/I ₂	15 days	13	—	—
Aqueous KI ₃	1 hr	28	40	—

The evidence that iodination in absolute ethanol can cause a marked increase in trypsin digestibility (Table 5) suggests that configurational changes have taken place in the proteins. Furthermore the X-ray diffraction evidence (Fraser and MacRae 1957) that iodination of tyrosine residues gives rise to a new sharp spot corresponding with a repeat along the meridian of 33 Å, together with sharpening of the spot of 25 Å and a noticeable decrease in sharpness of the high angle pattern (MacRae, personal communication), indicates in a positive manner an association of tyrosine with the crystalline regions of the fibre and suggests some disordering of the α -helices. There is therefore no sound basis for the assumption that tyrosine residues occur only in the amorphous regions of the fibre. The analogy with silk which Ghosh, Holker, and Speakman (1958) use is unsound since the crystallites of wool are largely in the form of α -helices whereas those of silk form β -sheets which do not accommodate tyrosine residues (Marsh, Corey, and Pauling 1956).

The location of the di-iodotyrosine residues in wool treated with propanolic iodine will be considered in a later paper.

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CHROMATOGRAPHIC FRACTIONATION OF THE ACETIC ACID SOLUBLE PROTEINS OF WHEAT FLOUR ON CARBOXYMETHYL-CELLULOSE

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Summary

The acetic acid soluble proteins extracted from wheat flour have been separated into 11 fractions by a chromatographic procedure using columns of carboxymethyl-cellulose, equilibrated with 0.005M acetate buffer, pH 4.1. Under these conditions one fraction passes unretarded through the column. Eight further fractions are eluted at pH 4.1 by employing a gradient to 0.2M NaCl in the presence of 1M dimethyl formamide (DMF) which prevents protein precipitation at ionic strengths greater than 0.03. The remaining two fractions are released from the column by stepwise changes of eluting solvent, the first with 0.005M acetate buffer containing 1.0M DMF and 0.5M NaCl, pH 4.1, and the second with a phosphate solution pH 12 (0.005M trisodium phosphate, 1.0M DMF, and 0.5M NaCl). The behaviour of four of the fractions on rechromatography suggests that they are distinct protein entities.

The acetic acid extracts of a series of flours with differing baking characteristics have been subjected to this chromatographic procedure. Very similar elution patterns are obtained from extracts of different samples of the same wheat variety; there are, however, qualitative differences between the patterns of extracts of flours milled from hard and soft wheats.

I. INTRODUCTION

Investigations of the composition and structure of wheat gluten by chemical and physicochemical techniques have led to a diversity of results depending upon the method used. Thus McCalla and co-workers (McCalla and Rose 1935; Spencer and McCalla 1938; McCalla and Gralen 1942), using sodium salicylate as a dispersing agent, considered gluten to consist of a very large number of fractions, each differing slightly in solubility and molecular weight. Other studies using the ultracentrifuge (Krejci and Svedberg 1935; Holme and Briggs 1959) have failed to show substantial resolution of either gluten or gliadin, about 90% of the material moving as a single peak. Heterogeneity with respect to charge has been well established by the moving boundary (Holme and Briggs 1959; Jones, Taylor, and Senti 1959), starch gel (Elton and Ewart 1960), and filter paper (Deschreider 1960; Zentner 1960) methods of electrophoresis.

It is clear that the classical concept of gluten as an association of two components, gliadin (alcohol soluble) and glutenin (alkali soluble) (Osborne 1907) is inadequate, and that further studies are required. While electrophoretic studies allow some assessment to be made of the heterogeneity of gluten, they do not permit the easy separation and large-scale purification of individual components afforded

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by chromatographic techniques. As indicated by Woychik, Dimler, and Senti (1960), a difficulty experienced with column chromatography of gluten in acid solution is its insolubility in the pH range 5–6 and in solutions of ionic strength greater than 0.03. This makes it impossible to use the commonly employed gradients of salt concentration or increasing pH for eluting adsorbed proteins from columns. Woychik, Dimler, and Senti (1960) overcame this difficulty in the chromatography of gluten on carboxymethyl-cellulose (CMC) by lowering, instead of raising the pH, and could obtain substantial resolution of five components by a stepwise elution procedure. By using dimethyl formamide (DMF) to maintain protein solubility, resolution of the acetic acid soluble extract of flour into six fractions by chromatography on CMC has been achieved with a conventional salt and pH gradient elution procedure (Simmonds and Winzor 1961).

This paper reports a refinement of this latter chromatographic technique which allows resolution of the acetic acid extracts of flours into 11 fractions. The results of a comparative study by this method of the acetic acid extracts of flours with widely differing baking characteristics are also discussed.

II. MATERIALS AND METHODS

(a) *Flour Samples*

The flours used were obtained from wheat samples sent to the Bread Research Institute of Australia for baking tests and physical testing; the samples were milled to approximately 70% extraction on a Buhler laboratory mill.

(b) *Extraction of the Acetic Acid Soluble Proteins*

The procedure used previously (Coates and Simmonds 1961; Simmonds and Winzor 1961) was followed throughout. In order to increase the concentration of protein being loaded onto the columns, only the first two acetic acid extracts were combined for chromatography. Prior to loading, the acetic acid extract was dialysed against 0.005M acetate buffer, pH 4.1 (0.005M sodium acetate, pH adjusted with acetic acid), and then clarified by centrifuging at 25,000 *g* for 15 min; 1–2% of the protein nitrogen was removed in this step.

(c) *Preparation of Columns for Chromatography*

CMC (Serva Entwicklungslabor, Hauserstrasse 45, Heidelberg, Germany, or Whatman powder CM 70) was treated with 0.1N NaOH followed by two washes with 0.05M acetate buffer, pH 4.1. After checking the pH of the supernatant liquid, the cellulose was poured into a column (1.5 × 15 cm), compacted by gentle air pressure, and washed thoroughly with 500 ml of 0.005M acetate buffer, pH 4.1.

Provision was made for six such columns to be set up simultaneously, each column receiving buffer via a separate micropump (Simmonds and Rowlands 1960) from a common reservoir. A gradient of increasing salt concentration was then introduced into this reservoir by connecting it to a second vessel filled with the limit buffer. Thus for columns with equal effluent flow rates the conditions could be varied in an identical manner for all six columns simultaneously. (The advantage

of this type of arrangement has been stressed by Marr and Gilbo 1960.) The flow rate of each column was adjusted to 1 ml/min by means of the micropumps, and 10-ml fractions were obtained by actuating the fraction collector at 10-min intervals. Chromatographic experiments were performed at room temperature (25–27°C).

(d) Chromatographic Procedure

100 ml of the dialysed extract, containing 40–100 mg of protein nitrogen, was applied to each column and washed in with 30 ml of 0.005M acetate buffer, pH 4.1, followed by 250 ml of the same buffer containing 1.0M DMF. A gradient, total volume 1 litre, with gradually increasing NaCl concentration was then employed as eluent, all other factors (pH, acetate, and DMF concentrations) being kept constant.

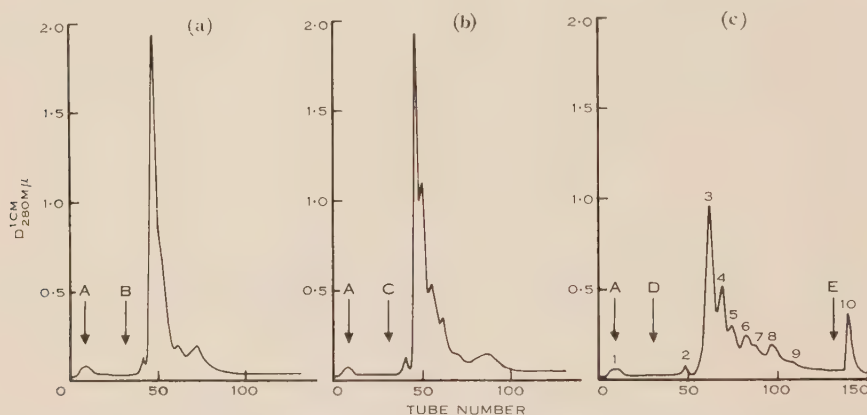


Fig. 1.—Effect of various salt gradients in the chromatography of the acetic acid extract of Glenwari flour on 1.5×15 cm columns of CMC. The protein was applied to the column in 0.005M acetate buffer, pH 4.1 (76 mg of protein nitrogen in each case). Legend for elution procedure: A, 0.005M acetate containing 1.0M DMF, pH 4.1; B, gradient started to 1.0M NaCl (in acetate-DMF); C, gradient started to 0.5M NaCl (in acetate-DMF); D, gradient started to 0.2M NaCl in acetate-DMF; E, acetate-DMF buffer containing 0.5M NaCl, pH 4.1. Size of fractions: 10 ml.

The procedure finally adopted used a linear gradient to 0.2M NaCl, followed by step-wise elution with 250 ml of the acetate-DMF buffer containing 0.5M NaCl (pH 4.1), and then 250 ml of a phosphate solution (0.005M trisodium phosphate, 1.0M DMF, 0.5M NaCl) the pH of which was adjusted to 12 with NaOH. The column was finally eluted with 400 ml of 0.1N NaOH.

(e) Protein Estimation

The amount of protein nitrogen applied to the columns was determined by a microKjeldahl method, and absorption measurements at 280 $m\mu$ were made on each effluent fraction as a measure of protein content. Since the turbid nature of the phosphate and NaOH fractions led to spurious absorption values, the effluent corresponding to each peak in the elution pattern was bulked and the relative amount of protein comprising each peak estimated on this bulked sample by the method of

Lowry *et al.* (1951). A sample of the dialysed flour extract of known nitrogen content was used as a standard for these estimations.

(f) *Rechromatography of Peaks*

The contents of tubes in the appropriate region of the effluent were combined and dialysed against the 0.005M acetate buffer before reloading on the CMC columns (1.5×15 cm). The effluent was collected in 5-ml fractions.

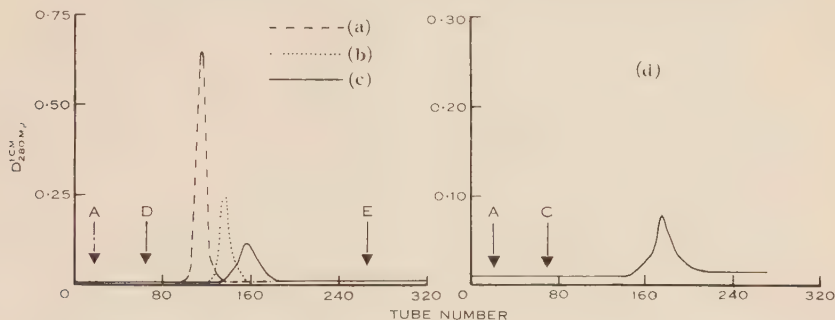


Fig. 2.—Rechromatography of four of the fractions obtained from the acetic acid extract of Glenwari flour by chromatography on CMC. Columns: 1.5×15 cm of CMC equilibrated to pH 4.1 with 0.005M acetate buffer. (a) Peak 3 (15 mg protein nitrogen); (b) peak 4 (8 mg); (c) peak 6 (6 mg); (d) peak 10 (1 mg). The arrows have the same significance as in Figure 1. Size of fractions: 5 ml.

III. RESULTS

(a) *Development of the Refined Technique*

The effect of varying the salt gradient is shown in Figure 1, which gives elution patterns from the chromatography of the acetic acid extract of an Australian wheat flour (Glenwari). The loading was 76 mg of protein nitrogen in each case. Figure 1(a) is the pattern obtained with a gradient to 1.0M NaCl (in 0.005M acetate – 1M DMF, pH 4.1), the conditions used previously (Simmonds and Winzor 1961). Figures 1(b) and 1(c) show the effect of reducing the concentration of NaCl in the limit solution of the gradient to 0.5M and 0.2M respectively. A noticeably better resolution is evident in (b) and is improved still further in (c). Using the area under the peaks as a measure of the amount of protein, the values (in arbitrary area units) are 150.2, 150.5, and 150.5 for (a), (b), and (c) respectively. The area under peak 10 in (c) (11.5) corresponds very closely with the area under the final peak (11.8) in (b).

(b) *Rechromatography of Peaks 3, 4, 6, and 10*

The patterns obtained on rechromatography of these fractions are shown in Figure 2. The symmetrical peak in Figure 2 (a) was obtained from peak 3, whilst that in Figure 2(b) was obtained after a second re-run of peak 4. Because of the small amount of protein available, peaks 6 and 10 have not been rechromatographed further than shown in Figures 2(c) and 2(d). In each experiment it should be mentioned that there is a small peak eluted with 0.1N NaOH. The ratios of the absorbances at 280 and 260 $m\mu$ for peaks 3 and 4 are 1.2 and 1.4 respectively.

TABLE 1
DISTRIBUTION OF PROTEIN IN THE CHROMATOGRAPHY ON CARBOXYMETHYL-CELLULOSE OF THE ACETIC ACID SOLUBLE PROTEINS OF WHEAT FLOUR

Wheat Variety	Nitrogen Content (g/100 g dry flour)	Baking Score*	Distribution (% of total protein)									
			I	II	III	IV	V	VI	VII	VIII	IX	X
Gabo	2.60	79	2	2	19	10	8	22	15	4	14	4
Gabo	2.21	76	1	1	19	9	7	22	13	5	18	5
Gabo	2.22	75	2	2	21	6	8	24	17	5	11	4
Gabo	1.79	70	1	2	18	10	6	23	14	6	16	6
Sabre	1.49	49	2	2	25	7	7	26	14	4	9	4
Unknown†	2.31	87	1	2	21	18	4	24	10	4	12	4
Glenwari	1.98	53	1	2	22	9	8	19	14	5	18	4
Glenwari	1.91	64	1	1	26	10	7	17	12	5	16	5
Insignia	2.06	79	2	2	20	15	8	12	17	5	11	8
Dural‡	2.92	51	2	2	19	6	6	21	13	3	23	7

* Maximum score 100. Details of score allocation are given by Coates and Simmonds (1961).

† Commercial flour—Manitoba No. 3 (Canada).

‡ Durum wheat.

(c) *Comparison of Flours by Column Chromatography*

The elution patterns for three different flours are illustrated in Figure 3. The most striking difference between them is the appearance of an extra peak in region III in the case of Gabo flour (Fig. 3(a)). The results of quantitative protein estimations by the Lowry method (Lowry *et al.* 1951) on samples obtained by bulking the contents of tubes as indicated in Figure 3, are given in Table 1 for all flours examined, together with wheat variety, baking score, and nitrogen content.

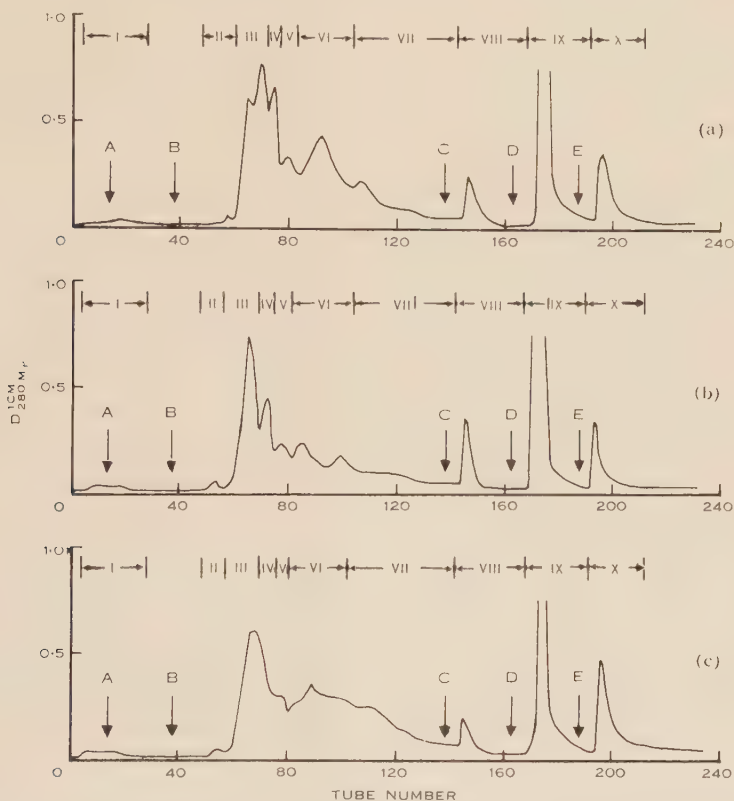


Fig. 3.—Elution patterns obtained in the chromatography of the acetic acid extracts of flours milled from three wheat varieties: (a) Gabo; (b) Glenwari; (c) Dural. Columns: 1.5×15 cm of CMC, equilibrated with $0.005M$ acetate buffer, pH 4.1. Legend for elution procedure: A, $0.005M$ acetate containing $1.0M$ DMF, pH 4.1; B, start of gradient to $0.2M$ NaCl in acetate-DMF buffer; C, $0.005M$ acetate buffer containing $1.0M$ DMF and $0.5M$ NaCl; D, $0.005M$ trisodium phosphate, $1.0M$ DMF, and $0.5M$ NaCl; E, $0.1N$ NaOH. Size of fractions: 10 ml.

IV. DISCUSSION

The chromatographic procedure described above has separated the acetic acid extracts of wheat flour into 11 fractions of differing chromatographic behaviour. Re-chromatography of four of the peaks has verified that these are definite chromatographic entities, which differ, moreover, in their ratio of absorbances at 280 and 260

m μ . It is not considered that the small amount of protein eluted by NaOH represents an additional component. It is more likely that either the reaction between protein and CMC is not completely reversible or that a small amount of denaturation has occurred. This peak has nevertheless been included in Figure 3 and Table 1 to indicate the amount of protein not removed from the column by the elution procedure employed.

If on rechromatography of these fractions the columns were eluted with the phosphate solution, pH 12, prior to NaOH, a small peak containing 3–5% of the applied protein appeared in the phosphate eluate and no protein was eluted by NaOH. The significance of the phosphate peak in experiments on crude flour extracts must therefore be questioned. However, the larger proportion of protein located in this peak in experiments on extracts (10–20% of total protein added) than that found when the major components are chromatographed separately suggests that additional proteins are also eluted by this solvent.

Previous chromatographic studies using CMC (Woychik, Dimler, and Senti 1960; Simmonds and Winzor 1961) have revealed only five and six components respectively in acetic acid extracts of wheat flour. The slower gradient employed in the present work undoubtedly explains the greater resolution obtained compared with that of Simmonds and Winzor (1961). A comparison with the work of Woychik, Dimler, and Senti (1960) is difficult because of differences between the elution procedures used. In both methods an acetic acid extract of flour was applied to the CMC in acid solution. However, the stepwise decreases in the pH of the eluting solvent used by Woychik, Dimler, and Senti (1960) may have tended to mask the presence of minor constituents.

The available data (Moore and Stein 1956; Hill, Kimmel, and Smith 1959; Cole 1960; Thompson and O'Donnell 1960) indicate that most successful chromatographic separations of proteins with similar isoelectric points have been achieved at a pH near the isoelectric points of the proteins. Although the conditions under which gluten is isoelectric are ill-defined (pH 5–7), greater resolution would be expected at the pH of this study, 4.1, than at the values 3.4–1.5 used by Woychik, Dimler, and Senti (1960).

The use of the higher pH values has necessitated the addition of DMF to all elution solvents in order to prevent precipitation of the flour proteins from solutions of ionic strength greater than 0.03. The use of such a reagent is assumed to effect only physical changes in proteins, and the possibility that these modifications lead to increased heterogeneity must be considered. The only well-characterized protein on which parallel chromatographic experiments have been performed to show the effect of such denaturing agents is ribonuclease, and the results are at first sight contradictory. Cole (1960) reported essentially identical elution patterns whether the chromatography was performed under the conditions described by Hirs, Moore, and Stein (1953) or in the same buffers containing 8M urea: in each case the same number of components was detected. On the other hand, Stark, Stein, and Moore (1960) observed that when ribonuclease solutions were maintained in 8M urea at 40°C, and then dialysed to remove urea, chromatography by the method of Hirs,

Moore, and Stein (1953) revealed a large *additional* peak in the elution curve. However, this peak was shown to have resulted from chemical modification of the ribonuclease with cyanate present in the 8M urea solution used. Since no chemically active impurity has yet been reported in DMF, it is assumed that the modification of the flour proteins has been purely physical.

Electrophoretic studies on gluten, which differs from the flour extract used here in that it apparently contains some water-soluble protein (Pence, Meeham, and Olcott 1956), have yielded widely differing estimates as to the exact number of components comprising the acid extract derived from either freshly prepared or freeze-dried gluten. Using the moving boundary method of electrophoresis, Holme and Briggs (1959) have detected three components in gliadin dispersed in an acetate-chloride buffer containing 3M urea (pH 3.8, ionic strength 0.10), while Jones, Taylor, and Senti (1959) found six components in gluten dissolved in acetate buffer (pH 3.1, ionic strength 0.03). Deschreider (1960) has reported resolution of lactic acid-propionic acid dispersions of gluten into four fractions by filter paper electrophoresis, this number being increased to seven if the filter paper curtain method is used (Zentner 1960). Eight components are indicated by starch gel electrophoresis (Elton and Ewart 1960). The 11 fractions detected in the present study of the acetic acid extracts of wheat flours can at best be regarded as a lower limit to the number of resolvable entities, since any heterogeneity in the material eluted by phosphate and NaOH would be concealed due to the stepwise-elution procedure used: the small amount of protein removed by the clarification step prior to chromatography has also to be considered as a possible source of further fractions.

The greater ability of chromatographic methods to reveal molecular species present in very minor amounts is well known: for example the heterogeneity in ribonuclease detected by these procedures (Hirs, Moore, and Stein 1953; Taborsky 1959) is barely detectable by moving boundary electrophoresis (Rothen 1940; Alberty, Anderson, and Williams 1948), which relies upon density-gradient stabilization at the sites of the boundaries. This method fails to detect species present in small amounts because of the gravitational instability of moving boundaries accompanied by too small density increments (Svensson 1960).

Shapira and Parker (1960) have separated by rechromatography several different fractions from chromatographically homogeneous ribonuclease which had been heated in solution. Their interpretation that some of the fractions are structural isomers of the same chemical entity would, if applicable to gluten, be a possible explanation of the observed heterogeneity. However, since single peaks have been obtained on rechromatography of several gluten fractions, it would be necessary to assume that interconversion between the various structural isomers does not occur, or is extremely slow. The only data available suggest that the action of hydrogen-bond-breaking agents on gluten is reversible (Cook and Alsberg 1931; McCalla and Rose 1935), and such isomers even if formed should thus be readily interconvertible. It would therefore seem that the simplest explanation of the fractionation results described is that several distinct components comprise the complete gluten complex. Some of these components may be stable aggregates resulting from thiol-disulphide interchange between the protein molecules comprising the gluten: exchange reactions

of this type are believed to occur during dough mixing (Goldstein 1957; Bloksma 1958; Mecham 1959; Frater, Hird, and Moss 1961) with the formation of a network of linked protein molecules throughout the dough. Minor differences in amide content, such as have been suggested as the basis of chromatographic inhomogeneity of myoglobin (Edmundson and Hirs 1961), could also account for some of the observed heterogeneity. Any conclusions as to the nature of the differences between components must be delayed until the individual fractions have been examined further.

The comparative study has not revealed striking differences between flours. In all cases there are three major fractions (columns III, VI, and IX in Table 1) and consideration of trends in the magnitude of these three allows division of the flours studied into three groups: (a) those in which $\text{III} > \text{VI} > \text{IX}$; (b) a group in which VI is the largest fraction; and (c) one flour (Dural) for which $\text{III} < \text{VI} < \text{IX}$. The three flours coming under category (a) are the soft wheats Glenwari and Insignia, while those in group (b) are milled from hard wheats. The allocation of the durum flour into a separate group is not surprising in view of its species difference. Since the nitrogen content of the flours used varied over a wide range (see Table 1), and the baking score is known to be strongly dependent on this quantity, no attempt has been made to correlate protein distribution between fractions with baking quality.

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SHORT COMMUNICATION

DISTRIBUTION OF GLUTAMYL TRANSFERASE IN THE RIPENING PEA SEED*

By A. H. G. C. RIJVEN†

Glutamyl transferase is considered identical with glutamine synthetase. Elliott (1953) achieved a 2000-fold purification of the enzyme from the germinating pea seed, and this material has formed a popular plant source of the enzyme.

In connection with a study of the localized distribution of the enzyme in the ripening grain of wheat, detailed in Rijven and Cohen (1961), it became of interest to learn whether a similar situation might be found in another developing seed.

The results of an experiment, in which pea seeds at different stages of ripeness were analysed are set out in Table 1.

TABLE 1

DISTRIBUTION OF GLUTAMYL TRANSFERASE ACTIVITY AND PROTEIN NITROGEN CONTENT IN THE
RIPENING PEA SEED

Fresh Weight per Seed (mg)	Glutamyl Transferase Activity (Δ O.D./hr)			Glutamyl Transferase Activity per Milligram Soluble Protein Nitrogen (Δ O.D./hr)		Protein Nitrogen Content (μ g)		
	Per Seed	Coat (%)	Embryo (%)	Coat	Embryo	Per Seed	Coat (%)	Embryo (%)
66.1	1.65					240		
222.6	4.68	96	4	24.2	2.6	612	72	28
345.0	5.97	93	7	33.8	1.2	1078	35	65
799.1	11.09	63	27	38.1	1.4	6673	8	92

For this experiment pods of *Pisum sativum* cv. Earlicrop were collected from the field, and the seeds divided into four groups of different fresh weights. The seeds, except for the smallest weight group, were divided into seed coat and embryo which includes the cotyledons. Extraction and assay procedures followed in general those described previously (Rijven and Cohen 1961). Again total protein nitrogen determinations were carried out on samples withdrawn before low- and after high-speed

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centrifugation. Specific activities were calculated with the soluble protein nitrogen values as basis, because the enzyme assays were on aliquots of the same high-speed supernatant.

The results show that the enzyme is localized predominantly in the seed coat tissues. On the seed basis, however, the activity in the embryo is substantial in the largest seed weight group, but it should be noticed that even there the specific activity in the coat remains many times higher than in the embryo; in fact in the latter it is no higher than in the endosperm of wheat. In contrast with wheat, disappearance of activity from the coat tissues must here coincide with the final maturation of the seed. This, together with the enormous accumulation of protein in the pea embryo, explains why in pea the total activity per seed rises continuously, whilst in wheat the activity per grain shows an early peak.

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INDEX

	PAGE		PAGE
<i>Acheta commodus</i> (Walk.), Action of Urea on Diapause in Eggs of	419	Bean Roots, Bioelectric Oscillations of. I	231
Adams, K. M., and Sobey, W. R.—		Bergersen, F. J.—	
Inheritance of Antibody Response. V. Correlated Antibody Responses to Various Related and Unrelated Antigens ..	594	The Growth of <i>Rhizobium</i> in Synthetic Media	349
Adams, K. M.—		Blunt, M. H.—	
<i>See also</i> Sobey, W. R.	588	<i>See</i> Evans, J. V. ..	87, 100
Antibody Response, Inheritance of. IV, V	588, 594	Braden, A. W. H.—	
Aphids, Transmission of Cauliflower Mosaic Virus by	187	<i>See</i> Ashton, G. C.	248
Ashton, G. C., and Braden, A. W. H.—		Briggs, M. H.—	
'Serum β -Globulin Polymorphism in Mice	248	The Visual Pigment of an Isopod Crustacean	487
Aspinall, D.—		Brock, R. D.—	
The Control of Tillering in the Barley Plant. I. The Pattern of Tillering and its Relation to Nutrient Supply	493	<i>See</i> Langridge, J.	66
Ballard, L. A. T.—		Burnet, F. M.—	
Studies of Dormancy in the Seeds of Subterranean Clover (<i>Trifolium subterraneum</i> L.). II. The Interaction of Time, Temperature, and Carbon Dioxide during Passage out of Dormancy	173	<i>See</i> Warner, N. L.	580
Barker, S.—		Carr, D. J., and Skene, K. G. M.—	
Studies on Marsupial Nutrition. III. The Copper-Molybdenum-Inorganic Sulphate Interaction in the Rottnest Quokka, <i>Setonix brachyurus</i> (Quoy & Gaimard)	646	Diauxic Growth Curves of Seeds, with Special Reference to French Beans (<i>Phaseolus vulgaris</i> L.) ..	1
Barley Plant, Control of Tillering in. I	493	Carr, D. J.—	
		<i>See also</i> Gaff, D. F.	299
		<i>See also</i> Skene, K. G. M. ..	13
		Cauliflower Mosaic Virus, Transmission of, by Aphids	187
		Cellobiase and Peroxidase, Coupling of, by Glucose Oxidase ..	489
		Cereal Proteins, Comparative Study of the Monolayers of ..	288
		<i>Chara australis</i> , Ionic Relations of Cells of. IV, V	26, 312
		Chick Embryo, Influence of Testosterone on Development of Bursa of Fabricius in	580
		Cohen, R.—	
		<i>See</i> Rijven, A. H. G. C. ..	552
		Crewther, W. G., and Dowling, L. M.—	
		Chemical Changes in Wool treated with Solutions of Iodine	677

	PAGE		PAGE
Cruickshank, I. A. M.—		Duloy, Margaret, and Mercer, F. V.—	
Germination of <i>Peronospora</i>		Studies in Translocation. I. The	
<i>tabacina</i> . Effect of Temperature	58	Respiration of the Phloem ..	391
Environment and Sporulation		Duloy, Margaret, Mercer, F. V.,	
in Phytopathogenic Fungi. II.		and Rathgeber, Nele—	
Conidia Formation in <i>Perono-</i>		Studies in Translocation. II.	
<i>spora tabacina</i> Adam as a Func-		Submicroscopic Anatomy of the	
tion of Temperature	198	Phloem	506
Cruickshank, I. A. M., and Perrin,			
Dawn R.—		<i>Erysiphe graminis tritici</i> , Additional	
Studies on Phytoalexins. III.		Resistance in <i>Triticum vulgare</i> to	70
The Isolation, Assay, and General		Evans, J. V.—	
Properties of a Phytoalexin from		Differences in the Concentra-	
<i>Pisum sativum</i> L.	336	tion of Potassium and the Type	
Cruickshank, I. A. M., and Rider,		of Haemoglobin between Strains	
N. E.—		and Sexes of Merino Sheep ..	274
<i>Peronospora tabacina</i> in Tobacco:		Evans, J. V., and Blunt, M. H.—	
Transpiration, Growth, and Re-		Electrolyte and Haematocrit	
lated Energy Considerations ..	45	Changes in the Blood of Sheep	
Dainty, J., and Hope, A. B.—		from Foetal to Postnatal Life ..	87
The Electric Double Layer and		Variation in the Gene Fre-	
the Donnan Equilibrium in		quencies of Potassium and	
Relation to Plant Cell Walls ..	541	Haemoglobin Types in Romney	
Day, M. F., and Venables, D. G.—		Marsh and Southdown Sheep	
The Transmission of Cauliflower		Established away from their	
Mosaic Virus by Aphids ..	187	Native Environment	100
Diauxic Growth Curves of Seeds,			
with Special Reference to French		Fisher, R. A.—	
Beans (<i>Phaseolus vulgaris</i> L.) ..	1	Possible Differentiation in the	
Dormancy in the Seeds of Sub-		Wild Population of <i>Oenothera</i>	
terranean Clover, Studies of ..	173	<i>organensis</i>	76
Dowling, L. M.—		Fowl Spermatozoa, Viability of, in	
See Crewther, W. G.	677	Dilute Suspension	637
Downes, A. M.—			
On the Mechanism of Incorpora-		Gaff, D. F., and Carr, D. J.—	
tion of [³⁵ S]Cystine into Wool	109	The Quantity of Water in the	
On the Amino Acids Essential		Cell Wall and its Significance ..	299
for the Tissues of the Sheep ..	254	Gene-Chromosome Configuration	
The Fate of Intravenous Doses		Effects, Accommodation of, in	
of Free and Plasma Protein-		Quantitative Inheritance and	
bound [³⁵ S]Cystine in the Sheep	427	Selection Theory	402
Downes, A. M., and Lyne, A. G.—		Gibberellin Content of Seeds of	
Studies on the Rate of Wool		<i>Phaseolus vulgaris</i>	13
Growth using [³⁵ S]Cystine .. .	120		

- | PAGE | PAGE |
|---|---|
| Goldacre, the Late P. L., and Unt, H.— | Jones, G.— |
| The Cultivation of Isolated Roots of Subterranean Clover and Effects of Amino Acids on their Growth Pattern | Estimates of Cortical Differentiation in Normal and "Doggy" Merino Wools |
| 323 | 485 |
| Griffing, B.— | Kerr, R. W.— |
| Accommodation of Gene-Chromosome Configuration Effects in Quantitative Inheritance and Selection Theory .. | Inheritance of DDT-resistance Involving the Y-Chromosome in the Housefly (<i>Musca domestica</i> L.) |
| 42 | 605 |
| Hill, A. V.— | Kindred, B. M.— |
| Dissemination of Conidia of <i>Peronospora tabacina</i> Adam .. | Abnormal Inheritance of the Sex-linked Tabby Gene |
| 208 | 415 |
| Hogan, J. P.— | A Maternal Effect on Vibrissa Score due to the Tabby Gene .. |
| The Absorption of Ammonia through the Rumen of the Sheep | 627 |
| 448 | Koch, Judith H., and Turner, Helen Newton— |
| Hogan, T. W.— | Studies on the Sodium-Potassium Balance in Erythrocytes of Australian Merino Sheep. I. Changes in Concentrations in the Erythrocytes of Lambs from Birth to 98 Days |
| The Action of Urea on Diapause in Eggs of <i>Acheta commodus</i> (Walk.) (Orthoptera: Gryllidae) | 79 |
| 419 | Koch, Judith H.— |
| Hog Thyroid Glands, Preparation of Pure Proteins from | <i>See also</i> Turner, Helen Newton |
| 475 | 260 |
| Hope, A. B.— | Langridge, J., and Brock, R. D.— |
| Ionic Relations of Cells of <i>Chara australis</i> . V. The Action Potential | A Thiamine-requiring Mutant of the Tomato |
| 312 | 66 |
| Hope, A. B.— | Larvae of Randomly Moving Insects, Distribution of .. |
| <i>See also</i> Dainty, J. | 598 |
| 541 | Lee, B. T. O., and Pateman, J. A.— |
| Hope, A. B., and Walker, N. A.— | Studies concerning the Inheritance of Ascospore Length in <i>Neurospora crassa</i> . I. Studies on Large-spored Strains |
| Ionic Relations of Cells of <i>Chara australis</i> R.Br. IV. Membrane Potential Differences and Resistances | 223 |
| 26 | Lyne, A. G.— |
| Jenkinson, I. S., and Scott, B. I. H.— | The Postnatal Development of Wool Follicles, Shedding, and Skin Thickness in Inbred Merino and Southdown-Merino Crossbred Sheep |
| Bioelectric Oscillations of Bean Roots: Further Evidence for a Feedback Oscillator. I. Extracellular Response to Oscillations in Osmotic Pressure and Auxin | 141 |
| 231 | Lyne, A. G.— |
| Jermyn, M. A.— | <i>See also</i> Downes, A. M. |
| The Coupling of Cellobiase and Peroxidase by Glucose Oxidase | 120 |
| 489 | <i>See also</i> Molyneux, G. S. |
| | 131 |

	PAGE
Mammals, Herbivorous, Technique for Ascertaining the Diet of	157
Marshall, D. C.—	
The Freezing of Plant Tissue ..	368
Marsupial Nutrition, Studies on.	
III	646
Mercer, F. V.—	
See Duloy, Margaret ..	391, 506
Mice, Serum β -Globulin Polymorphism in	248
Molyneux, G. S.—	
A Morphological and Histochemical Study of the Bacterial Degradation of Wool Fibres <i>in vivo</i>	440
Molyneux, G. S., and Lyne, A. G.—	
Studies on Experimental Dermal Cysts in Sheep	131
Mouse Coat, Growth of. VIII ..	620
<i>Musca domestica</i> L., Inheritance of DDT-resistance in	605
Nay, T.—	
Growth of the Mouse Coat. VIII. Changes in the Coat and Body Weight under Heat Stress	620
<i>Neurospora crassa</i> , Inheritance of Ascospore Length in. I ..	223
<i>Nippostrongylus muris</i> (Yokogawa), Pathology of Infestation of the Rat with	165
O'Brien, T. P., and Wardlaw, I. F.—	
The Direct Assay of ^{14}C in Dried Plant Materials	361
O'Donnell, I. J., and Thompson, E. O. P.—	
Studies on Oxidized Wool. IV. Fractionation of Proteins Extracted from Wool on DEAE-Cellulose using Buffers containing 8M Urea	461

	PAGE
<i>Oenothera organensis</i> , Possible Differentiation in the Wild Population of	76
Pateman, J. A.—	
See Lee, B. T. O.	223
Paul, Jean I.—	
The Nitrogen Requirements of some Members of the Viridans Group of Streptococci	567
<i>Peronospora tabacina</i> , Conidia Formation in, as a Function of Temperature	198
<i>Peronospora tabacina</i> , Dissemination of Conidia of	208
<i>Peronospora tabacina</i> , Effect of Temperature on Germination of	58
<i>Peronospora tabacina</i> in Tobacco ..	45
Perrin, Dawn R.—	
See Cruickshank, I. A. M. ..	336
<i>Phaseolus vulgaris</i> , Diauxic Growth Curves of Seeds of	1
<i>Phaseolus vulgaris</i> , Quantitative Study of the Gibberellin Content of Seeds of	13
Phytoalexins, Studies on. III ..	336
<i>Pisum sativum</i> L., Isolation of a Phytoalexin from	336
Plant Cell Wall, Quantity of Water in, and its Significance	299
Plant Cell Walls, Electric Double Layer and the Donnan Equilibrium in Relation to	541
Plant Materials, Dried, Direct Assay of ^{14}C in	361
Plant Tissue, Freezing of	368
Pugsley, A. T.—	
Additional Resistance in <i>Triticum vulgare</i> to <i>Erysiphe graminis tritici</i>	70
Rat, Pathology of Infestation with <i>Nippostrongylus muris</i>	165
Rathgeber, Nele—	
See Duloy, Margaret	506

	PAGE		PAGE
Rhizobium, Growth of, in Synthetic Media	349	Follicles, Shedding, and Skin Thickness in	141
Rider, N. E.—		Sheep, Southdown, Variations in the Gene Frequencies of Potassium and Haemoglobin Types in	100
<i>See</i> Cruickshank, I. A. M. ..	45	Sheep, Studies on Experimental Dermal Cysts in	131
Rijven, A. H. G. C.—		Shulman, S., and Stanley, P. G.—	
Distribution of Glutamyl Transferase in the Ripening Pea Seed	700	Preparation of Pure Proteins from Hog Thyroid Glands by Column Chromatography on Diethylaminoethyl-cellulose ..	475
Rijven, A. H. G. C., and Cohen, R.—		Simmonds, D. H., and Winzor, D. J.—	
Distribution of Growth and Enzyme Activity in the Developing Grain of Wheat	552	Chromatographic Fractionation of the Acetic Acid Soluble Proteins of Wheat Flour on Carboxymethyl-cellulose ..	690
Schinckel, P. G.—		Skene, K. G. M., and Carr, D. J.—	
Mitotic Activity in Wool Follicle Bulbs	659	A Quantitative Study of the Gibberellin Content of Seeds of <i>Phaseolus vulgaris</i> at Different Stages in their Development ..	13
Scott, B. I. H.—		Skene, K. G. M.—	
<i>See</i> Jenkinson, I. S.	231	<i>See also</i> Carr, D. J.	1
<i>Setonix brachyurus</i> (Quoy & Gaimard), Copper-Molybdenum-Inorganic Sulphate Interaction in Nutrition of	646	Slatyer, R. O.—	
Sheep, Absorption of Ammonia through the Rumen of	448	Effects of Several Osmotic Substrates on the Water Relationships of Tomato	519
Sheep, Amino Acids Essential for the Tissues of	254	Sobey, W. R., and Adams, K. M.—	
Sheep, Australian Merino, Studies on the Sodium-Potassium Balance in Erythrocytes of. I, II ..	79, 260	Inheritance of Antibody Response. IV. Heritability of Response to the Antigens of <i>Rhizobium meliloti</i> and Two Strains of Influenza Virus ..	588
Sheep, Electrolyte and Haematocrit Changes in the Blood of, from Foetal to Postnatal Life ..	87	Sobey, W. R.—	
Sheep, Fate of Intravenous Doses of Free and Plasma Protein-bound [³⁵ S]Cystine in	427	<i>See also</i> Adams, K. M.	594
Sheep, Merino, Differences in the Concentration of Potassium and the Type of Haemoglobin between Strains and Sexes of ..	274	Stanley, P. G.—	
Sheep, Romney Marsh, Variations in the Gene Frequencies of Potassium and Haemoglobin Types in	100	<i>See</i> Shulman, S.	475
Sheep, Inbred Merino and Southdown-Merino Crossbred, Postnatal Development of Wool		Storr, G. M.—	
		Microscopic Analysis of Faeces, a Technique for Ascertaining the Diet of Herbivorous Mammals	157

	PAGE		PAGE
Subterranean Clover, Cultivation of Isolated Roots of	323	Viridans Group of Streptococci, Nitrogen Requirements of ..	567
Symons, L. E. A.—		Wales, R. G., and White, I. G.—	
Pathology of Infestation of the Rat with <i>Nippostrongylus muris</i> (Yokogawa). VI. Absorption <i>in vivo</i> from the Distal Ileum ..	165	The Viability of Fowl Spermatozoa in Dilute Suspension ..	637
Tabby Gene, Abnormal Inheritance of the Sex-linked ..	415	Walker, N. A.—	
Tabby, Gene, Maternal Effect on Vibrissa Score due to the ..	627	<i>See</i> Hope, A. B.	26
Thompson, E. O. P.—		Wardlaw, I. F.—	
<i>See</i> O'Donnell, I. J.	461	<i>See</i> O'Brien, T. P.	361
Tomato, A Thiamine-requiring Mutant of	66	Warner, N. L., and Burnet, F. M.—	
Tomato, Effects of Several Osmotic Substrates on the Water Relationships of	519	The Influence of Testosterone Treatment on the Development of the Bursa of Fabricius in the Chick Embryo	580
Translocation, Studies in. I, II 391, 506		Wheat Flour, Chromatographic Fractionation of the Acetic Acid Soluble Proteins of	690
<i>Trifolium subterraneum</i> L., Studies of Dormancy in the Seeds of ..	173	Wheat Gluten, Comparative Study of the Monolayers of	288
<i>Triticum vulgare</i> , Additional Resistance in, to <i>Erysiphe graminis tritici</i>	70	White, I. G.—	
Tschoegl, N. W.—		<i>See</i> Wales, R. G.	637
A Comparative Study of the Monolayers of Various Cereal Proteins and of Wheat Gluten of Differing Characteristics ..	288	Williams, E. J.—	
Turner, Helen Newton, and Koch, Judith H.—		The Distribution of Larvae of Randomly Moving Insects ..	598
Studies on the Sodium-Potassium Balance in Erythrocytes of Australian Merino Sheep. II. Observations on Three Merino Strains	260	Winzor, D. J.—	
Turner, Helen Newton—		<i>See</i> Simmonds, D. H.	690
<i>See also</i> Koch, Judith H. ..	79	Wool Fibres, Morphological and Histochemical Study of the Bacterial Degradation of ..	440
Unt, H.—		Wool Follicle Bulbs, Mitotic Activity in	659
<i>See</i> Goldacre, the Late P. L. ..	323	Wool Follicles, Postnatal Development of	141
Venables, D. G.—		Wool Growth, Studies on Rate of ..	120
<i>See</i> Day, M. F.	187	Wool, Mechanism of Incorporation of [³⁵ S]Cystine into	109
		Wool, Normal and "Doggy" Merino, Estimates of Cortical Differentiation in	485
		Wool, Oxidized, Studies on. IV ..	461
		Wool treated with Solutions of Iodine, Chemical Changes in ..	677